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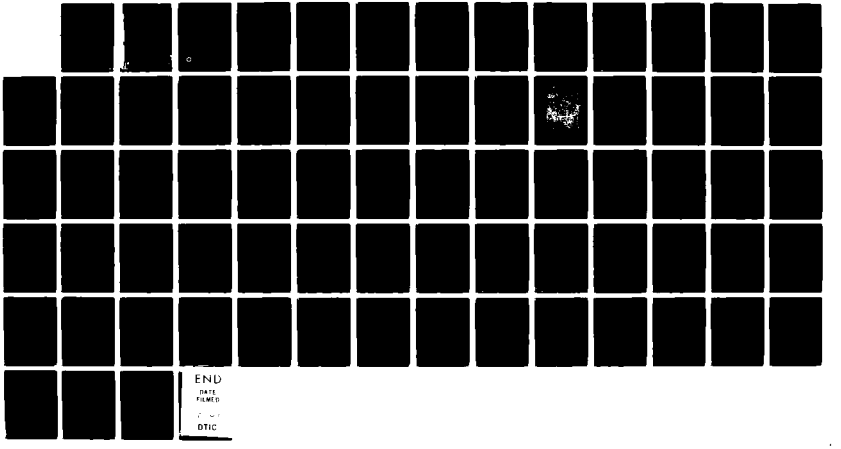

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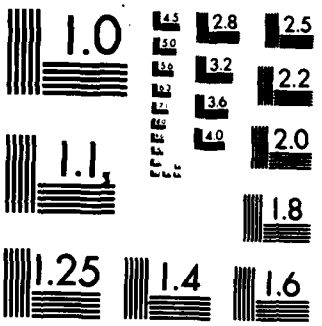

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Task No. NR 205-039

ANNUAL TECHNICAL REPORT NUMBER 3

HORMONAL INTERFERENCE WITH PHEROMONE SYSTEMS IN PARASITIC ACARINES, ESPECIALLY IXODID TICKS

By

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This report was supported in part by the Office of Naval Research,
Microbiology Program, Naval Biology Project,
under Contract No. N00014-80-C-0546, NR 205-039.

Submitted by the
Old Dominion University Research Foundation
P. O. Box 6369
Norfolk, Virginia 23508

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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER 3	2. GOVT ACCESSION NO. AD-A128 779	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Hormonal Interference with Pheromone Systems in Parasitic Acarines, Especially Ixodid Ticks		5. TYPE OF REPORT & PERIOD COVERED Annual, May 1, 1982 to April 30, 1983 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Daniel E. Sonenshine, Principal Investigator, Drs. P.J. Homsher & J.H. Oliver, Co-Investigators		8. CONTRACT OR GRANT NUMBER(s) N00014-80-C-0546
9. PERFORMING ORGANIZATION NAME AND ADDRESS Department of Biological Sciences Old Dominion University, Norfolk, VA 23508		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS Naval Biology NR 205-039
11. CONTROLLING OFFICE NAME AND ADDRESS Microbiology, Naval Biology Office of Naval Research Arlington, VA 22217		12. REPORT DATE May, 1983 13. NUMBER OF PAGES
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office) ONR Resident Representative George Washington University, 2216 G St., N.W. Washington, D.C. 20037		15. SECURITY CLASS. (of this report) Unclassified 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

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17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

Hormones; Ecdysteroids; Pheromones; Genital Pheromone(s); Precocene-2; Hyalomma dromedarii; Dermacentor variabilis; Radioimmunoassay; juvenile hormone; Dermanyssus gallinae

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Hormones; Edcysteroids; Pheromones; Genital Pheromone(s); Precocene-2; Hyalomma dromedarii; Dermacentor variabilis; Radioimmunoassay; juvenile hormone; Dermanyssus gallinae.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

New evidence which confirms the existence of ecdysterone (beta-ecdysone) in the ticks Dermacentor variabilis (Say) and Hyalomma dromedarii Koch is presented. Evidence implicating the occurrence of alpha-ecdysone, in much smaller amounts than that observed for ecdysterone, is also noted. Polar compounds, at least two of which react in the radioimmunoassay for ecdysone, are also present in both species, and are probably metabolites of the active hormones. No consistent relationship was found in the changes in ecdysteroid concentrations during development, maturation or feeding and the onset of sex pheromone

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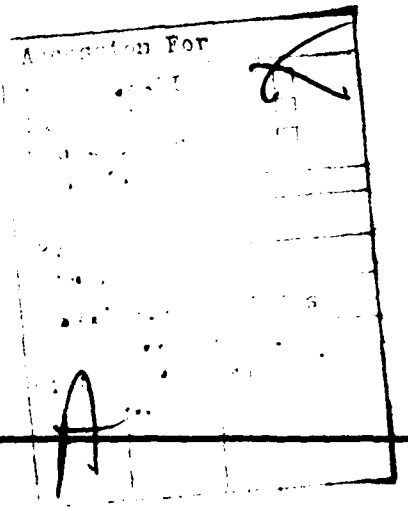
activity. However, ultrastructural changes in the foveal glands following exposure to exogenous ecdysterone parallel those seen in females secreting pheromone. Additional evidence of direct excitation of sex pheromone activity was found in H. dromedarii exposed to exogenous ecdysterone or an ecdysterone analog. The effects of the analog were especially noteworthy, specifically the excitation of sex pheromone production in male H. dromedarii and acceleration of ecdysis. The finding of increased 2,6-dichlorophenol content in female H. dromedarii exposed to exogenous ecdysterone confirms earlier evidence (reported in the previous annual progress report) of an excitatory role for this hormone. The discovery of incorporation of ^{14}C labelled carbons from labelled cholesterol into ecdysterone by H. dromedarii provides additional evidence of an natural, biosynthetic mechanism in this species. Autoradiographs of the ^{14}C cholesterol treated ticks suggest extensive incorporation of this material in the gonads, as well as significant incorporation in the synganglion (cortical zone) and foveal glands. No evidence of significant transfer of ecdysones from the parental to the F^1 generation was observed. Overall, the accumulating evidence suggests that ticks have the enzyme systems and receptor mechanisms to produce, degrade, and respond to ecdysones in a manner generally similar to that observed in insects. In addition, there is important evidence that ecdysones directly affect sex pheromone activity in H. dromedarii.

Studies on the occurrence of genital sex pheromones were continued. Male perception of an excitatory substance detected in the female, presumably near or in the gonopore, is reviewed.

The effects of the anti-allatotropin Precocene-2 and the juvenile hormone, JH3, on reporduction in the chicken mite, Dermanyssus gallinae is described. These studies are part of a continuing, long term effort to determine the existence and biological roles of JH-like compounds, if any, in the Acari. Precocene-2 was found to have negative effects on oogenesis, oviposition, and hatchability of the offspring. Evidence is presented which suggests that the effects are temporary and that treated mites fed subsequent blood meals recover regarding all three parameters. When Precocene-2 treated mites were also treated with juvenile hormone (JH 3), egg production increased as compared to mites exposed only to Precocene-2. However, JH treatment was only partially successful in reversing the effects of Precocene-2, and complete recovery of ovipositional ability was not achieved. Nevertheless, the findings provide evidence of juvenile hormone-like activity in these mites.



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I. Introduction

This project was designed to investigate hormonal regulation of mating behavior in ticks as well as reproductive strategies in the Acari. Recent evidence, including new findings reported for the first time in this report, have demonstrated the presence of several of the same hormones in ticks as are found in insects. The presence of others is implied by experimental studies. Insect and tick hormonal systems appear increasingly similar, although important differences may remain to be discovered. This similarity should be considered in interpreting the significance of the findings reported in the following pages.

Substantial evidence of hormonal excitation of sex pheromone activity has now been obtained. In vivo studies, reported at the VIth International Congress of Acarology (Dees et al. in press), demonstrated that exogenous ecdysterone introduced in physiological doses, excited sex pheromone activity. This was confirmed in more recent studies done during the current project year, which also suggested that the threshold for sex pheromone activity is much lower than that necessary to accelerate ecydsis. In addition, direct evidence of the presence of ecdysterone, small amounts of ecdysone, and other unknown ecdysteroids in Hyalomma dromedarii and Dermacentor variabilis was obtained. Another similarity with insects was the finding that ecdysterone is synthesized from cholesterol. Studies with exogenous JH were done also, but no evidence of a juvenilizing effect or disruption of sex pheromone activity was found.

Other studies in this report describe continuing research on the genital sex pheromones of ticks, particularly the role of the cheliceral digits in pheromone perception.

Collaboration among the several laboratories participating in the project was continued, namely, between (1) the Medical Zoology Department, U.S. NAMRU-3, Cairo, Egypt, (2) The Department of Biological Sciences, Old Dominion University, Norfolk, Virginia, and (3) the Department of Biology, Georgia Southern College, Statesboro, Georgia. In addition, studies on aspects of pheromone chemistry were done by the Department of Chemistry,

College of Environmental Sciences and Forestry, State University of New York, Syracuse, New York. Throughout the report, an effort is made to give due credit to the work done at the various collaborating laboratories.

This report is concerned almost exclusively with the results of studies done at Old Dominion University and Georgia Southern College. Reference, however, is made to studies done primarily at NAMRU-3, which will be reported in detail elsewhere.

II. Ecdysteroids in the American Dog Tick, Dermacentor variabilis(Say) During Different Periods of Tick Development (Acari:Ixodidae) (Old Dominion University)

Introduction

Ecdysteroids represent a class of steroid hormones which occur in many if not all forms of arthropods. Ecdysteroids regulate the molting process (Riddiford and Truman, 1978), vitellogenesis (Borovsky, 1981; Hagedorn et al. 1975), diapause termination (Bradfield and Denliger, 1980) and selected developmental processes (Ryerse, 1980). These hormones also occur in ticks. Solomon et al. (1982) summarize experimental evidence implicating their presence. Other workers used chemical techniques (Bouvier et al. 1982) on a combination of chemical methods and radioimmunoassay (RIA) to demonstrate ecdysteroids in ixodid ticks (Delbecque et al. 1978; Dees et al. 1983; Diehl et al. 1982), and the argasid tick Ornithodoros moubata (Murray) (Germond et al. 1982). The latter was found to be capable of metabolizing ecdysone to ecdysterone as well as other, unidentified metabolites (Bouvier et al. 1982).

Clearly, ecdysteroids and enzymes which act on them appear to be widespread in ticks. Moreover, the tick hormones appear to be similar to those of insects. Both alpha and beta ecdysones have been reported in Amblyomma hebraeum Koch (Delbecque et al. 1978) and O. moubata Bouvier et al. 1982). However, the occurrence of other ecdysteroids is not excluded. Indeed, one or more of the ecdysteroid "metabolites" described by Bouvier and his colleagues may represent other, active hormones of this class.

This paper describes the results of studies to determine whether ecdysteroids occur in the American dog tick, Dermacentor variabilis(Say), the major vector of Rocky Mountain spotted fever in the eastern United States. We were especially interested in determining the ecdysteroid concentrations at different periods of tick development, and in comparing these concentrations with important physiological events, namely, molting, feeding, and

sex pheromone activity. Excitation of sex pheromone activity was reported in Hyalomma dromedarii Koch, exposed to exogenous ecdysterone (Dees et al. in press). Although conclusive identification of the different ecdysteroids was not attempted, studies to determine the variety of RIA positive steroids during different tick developmental periods was done.

Materials and Methods

Ticks. The American dog tick, D. variabilis, was colonized and reared as described previously (Sonenshine et al., 1977). Immature ticks were allowed to feed on albino rats (Rattus norvegicus), adults on rabbits (Oryctalagus cuniculus). Ticks were held in an Aminco Climate Lab environmental chamber at 27° ± 0.5°C and 90 ± 2% RH during their non-parasitic periods.

Tick extracts. The materials used for radioimmunoassay (RIA) are listed in table 1. Extracts of each population were prepared by homogenizing the tick material with a Ten Broeck glass homogenizer (American Scientific Products, Columbia, MD) in methanol and water (65:35, v/v). The mixture was freeze-thawed, centrifuged, and washed 2 X with this same solvent. The precipitate was discarded, and the dried sediment re-extracted 3 X with 100% reagent grade methanol (J. T. Baker Co., Phillipsburg, NJ) with vigorous shaking. The methanol extract was centrifuged to remove extraneous material, dried, re-extracted 4 X with 50% methanol/benzene, again with 25% methanol/benzene, and finally, reconstituted in 100% ethanol. The final extract was stored (-20°C.) until needed for assay. Samples of these extracts were also assayed by High Performance Liquid Chromatography, as described below. The putative ecdysteroids were collected, and the fractions were assayed by RIA.

The tick material used to determine the presence of sex pheromone, 2,6-dichlorophenol (2,6-DCP) is listed in table 2. The tick material was extracted in double-distilled hexane Omni- Solv (Krackler Chemical Co., Albany NY) as described previously (Sonenshine et al. 1977; Sonenshine et al. 1982).

Table 1. Ecdysteroid concentrations in Dermacentor variabilis during different periods of tick development.

Life Stage	Days Post-Eclosion/ Feeding/ovi	No. in Sample	Ecdysteroid Conc. pg/tick	pg/mg
embryos	0	14,659	5.22 ± 2.51	68.63 ± 32.99
"	7	15,077	8.66 ± 5.46	113.84 ± 71.78
"	14	15,797	81.16 ± 13.64	1066.89 ± 0.89
unfed larvae	21	111,423	1.15 ± 0.31	34.66 ± 9.34
engorged larvae	0	4,000	12.56 ± 3.68	24.64 ± 7.22
" "	2	4,000	23.52 ± 8.92	49.4 18.77
" "	5	4,000	19.53 ± 4.94	44.(11.26
" "	8	4,000	13.19 ± 4.79	32.5 11.83
unfed nymphs	21	7,595	1.71 ± 1.20	11.7 8.21
engorged nymphs	0	100	21.32 ± 7.02	1.41 ± 0.46
" "	5	100	394.74 ± 156.56	34.57 ± 10.33
" "	10	100	973.50 ± 297.69	85.25 ± 26.07
" "	15	100	822.93 ± 296.41	77.42 ± 27.88
unfed males	0	20	386.00 ± 122.0	54.37 ± 17.18
" "	15	20	38.00 ± 3.0	8.52 ± 0.67
fed males	8	20	131.67 ± 2.36	12.54 ± 0.23
unfed female	0	20	722.4 ± 198.6	76.85 ± 21.1
" "	5	20	476.68 ± 24.9	60.26 ± 3.15
" "	10	20	156.40 ± 32.0	23.00 ± 4.71
" "	15	20	150.00 ± 2.4	6.65 ± 2.3

Table 1. Ecdysteroid concentrations in Dermacentor variabilis during different periods of tick development. (concluded)

Life Stage	Days Post-Eclosion/ Feeding/ovip	No. in Sample	pg/tick	pg/mg
part-fed females	1	20	193.3 ± 55.5	35.47 ± 10.18
"	2	20	168.5 ± 27.6	27.53 ± 4.51
"	4	20	293.8 ± 71.5	26.95 ± 6.56
"	7	20	6,783.3 ± 493.3	79.7 ± 5.80
part-fed females 1 day post-mating	8	20	3,431.1 ± 622.2	8.45 ± 1.53
Replete females (day of drop-off)	0	20	8,782.5 ± 3,834.4	16.96 ± 7.40
Ovipos. female	2	15	18,772.2 ± 3,511.5	91.17 ± 17.05

Table 2. Changes in the concentration of sex pheromone, 2,6-dichlorophenol, in unfed and feeding Dermacentor variabilis females.

Days	No. in Sample	ng DCP
Post-molting		
0	100	0.0
5	100	0.6
10	100	2.6
15	100	1.1
Feeding		
1	67	2.1
2	63	3.6
4	95	3.5
7	211	4.2
8	46	0.2
(1 day post-mating)		

Radioimmunoassay (RIA). The Horn I-1 anti-ecdysone anti-serum used in these tests was obtained (as a lyophilized powder) from Dr. J. D. O'Connor, University of California, Los Angeles. It was reconstituted in distilled water and stored frozen (-65°C) until needed. Aliquots of the tick extracts (methanol) described above were dried under nitrogen and reconstituted in 100 ul borate buffer. These were mixed with 50 ul aliquots (also in borate buffer) of tritiated alpha-ecdysone (16,000 DPM) (New England Nuclear Corp., Boston, MA, 80 Ci/mmol) and 50 ul of antiserum. The mixture was incubated for 24 hours at room temperature. After cooling (4°C), 200 ul of a saturated ammonium sulfate solution was added to each tube. The proteins were allowed to precipitate overnight and the solution was vortexed and centrifuged at 2500 rpm. After centrifugation, the supernatant was removed, the precipitate resuspended in fresh borate buffer:SAS solution (1:1), and the mixture centrifuged again. The supernatant was removed and the precipitate dispersed in 25 ul 100% ethanol plus 600 ul RIA Flour (New England Nuclear Corp., Boston, MA) in minivials. Duplicate samples were prepared for each extract and each dilution of that extract. Standards were prepared in a similar manner using known quantities ranging from 25 pg/ul to 1000 pg/ul of authentic alpha-ecdysone (Sigma Chemical Co., St. Louis, MO). Radioassay was done with a Beckman Model LS 250 Liquid scintillation counter. Counting efficiency after quench correction (external standard method) was 29%.

The purity of the ^{3}H alpha-ecdysone was confirmed by thin-layer chromatography (TLC) before use in the various tests (see below).

Chromatography (HPLC). The chemical characteristics of the ecdysteroids in the tick extracts were determined by TLC and High Pressure Liquid Chromatography (HPLC). TLC was done on Whatman K5WF thin layer silica gel plates (Whatman, Inc., Clifton, NJ) in chloroform:methanol (4:1, v/v). HPLC was done with a Waters System comprising Model 6000 and M45 pumps, Model 720 system controller, 730 Data Module, Model u6K injector, and a model 440 UV absorbance detector (Waters, Inc., Milford, MA). The column was a model RCM, 8 mm I. D, 10uM MicroBondapax C-18 column. The solvent system was methanol:water:acetic acid (65:35:1), v/v) and the flow

rate was 1.5 ml/min. In later studies, the column was a 10 uM Radial Pak C-18 column, 5 mm. x 10 cm.; a 5 uM MicroBondapax C-18 column, 5 mm. x 10 cm. was also used. The same solvent system was used but the ratio of the solvents was reduced to 45:55:1, and the flow rate was reduced to 1.0 ml/min. Samples selected for analysis consisted of aliquots of 50-100 ul of the original extract of the different life stages assayed by RIA (table 1), and reconstituted in reagent grade methanol; 10 ul aliquots were injected into the HPLC. Aliquots of each extract were injected at least 3 times. The ecdysteroids in the tick extracts were identified by comparison of their retention times with authentic insect ecdysones and by radioimmunoassay of the specific isolates. The amounts of each RIA postive compound were determined by averaging the amounts found in the 3 injections of each sample.

Sex pheromone content (GLC). To determine the amounts of sex pheromone in the ticks, hexane extracts of tick material were passed through precleaned Sep Paks (Waters and Associates, Inc., Milford, MA) containing Florasil to separate the 2,6-DCP from other organic materials. The Sep Paks were rinsed with fresh hexane (3 X) to release residual non-polar molecules, then rinsed with a mixture of 1:1 diethyl ether:petroleum ether to release the phenol. Recovery with this technique is approximately 100%, as confirmed with tests with known standards. Aliquots of the final extract were injected into a Shimadzu GC-6A gas chromatograph (Shimadzu, Inc., Rockville, MD) using Hamilton Model 701-RN microliter syringes with Chaney adapter and 26 S gauge needles. The gas chromatograph was equipped with a constant-current electron capture (EC) detector with a ^{63}Ni foil to detect halogenated compounds. Detector and injection port temperatures were 250°C, the column was 200°C. and the flow rate for the carrier gas (N2) was 75 ml/min. The column was a 0.6 cm O.D. x 56 cm long curved glass tube packed with Tenax 80/100 absorbent. Recordings and modification of compounds were made with a Shimadzu C-R1A Chromatopac Multiprocessor (Shimadzu, Inc., Rockville, MD) connected to the gas chromatograph.

Identification of 2,6-DCP in the extracts was done by comparing sample compound and authentic 2,6-DCP retention times, and by coinjection of the authentic standard and observing that it co-chromatographed with the sample compound.

Ultrastructure. To assess the effect of exogenous beta-ecdysone on pheromone gland ultrastructure, the foveal glands of unfed females

inoculated (as engorged nymphs on the day of drop off) with 10 ng beta-ecdysone were removed. Controls were inoculated with (also as engorged nymphs) with 1% saline. The tissues were fixed in cold (4°C) 4% glutaraldehyde (with 0.1 M S-Collidine buffer, pH 7.4), dehydrated and, embedded in Epon in accordance with previously described electron microscopy techniques (Dawes, 1971). Following hardening, thin (600 A) sections were cut using an LKB Ultramicrotome III (LKB Instruments, Inc., Rockville, MD). The thin sections were mounted on uncoated copper grids, and stained with saturated uranyl-acetate and lead citrate. The stained sections were viewed using a Hitachi HU-11B transmission electron microscope.

Results

Evidence of the existence of ecdysteroids (ECD) in D. variabilis was obtained by radioimmunoassay. ECD's were found in all life stages, from embryos to and including replete females (table 1). When measured in concentrations per individual, the lowest concentrations were found in the unfed larvae and unfed nymphs. ECD concentrations were found to increase during periods of development, i.e., embryogenesis and molting. In the embryos, the ECD concentration rose sharply on day 14, approximately 5 or 6 days prior to hatching. In the larvae, the ECD concentration rose approximately 12 fold during feeding and continued to increase during the post-engorgement molting process. Although the peak concentration was reached on day 2 post-dropping, the ECD concentration remained high through day 5 post-dropping, and the differences were not significant (p 0.1, 2 d. f., n. s.). A similar pattern was found in the feeding and molting nymphs. In this case, however, the increases were much greater than in the larvae, reaching almost 1 ng by day 10 post-dropping. Total ECD content in the engorged nymphs reached concentrations more than 40 times that found in the engorged larvae. Following molting, the ECD concentration declined in the unfed adults, to only 37 pg or 38 pg/tick by day 15 in females and males, respectively. With the commencement of feeding, the ECD concentration rose again, increasing more than 5 fold in the first day, then changing only slightly until day 7. A tremendous increase in ecdysteroid activity was observed on feeding day 7, to 6.783 ng/tick, or approximately 183 times the amount found in the unfed females. Similar trends are observed when the ECD concentrations are measured in terms picograms/milligram of tick material;

however, the highest concentrations are found in the embryos, while the concentrations immediately following drop off (completion of feeding) appears to have declined, perhaps a reflection of the massive dilution with blood and other fluids that occurs at this stage. The sharp rise in ECD concentration during female feeding is also evident, but the magnitude of the rise is not as great as that reflected in the pg/tick values.

Chromatography. Assay of the extracts of D. variabilis nymphs revealed the presence of at least 8 compounds (fig. 1). When isolated and assayed by RIA, 3 of the 8 fractions reacted with the Horn I-1 alpha ecdysone antiserum. Two of the fractions, fractions 1 and 2, had retention times which were very different from that of beta ecdysone. The 3rd fraction was identified as beta-ecdysone (ecdysterone) by the fact that it co-chromatographed with the authentic standard. A fourth fraction, fraction 4, had a retention time near 2-ecdysone (within 0.1 min.) and is probably this compound. It was present in only trace amounts and frequently could not be detected at all. Three relatively polar fractions were not RIA positive and did not occur consistently in all assays. Two relatively apolar fractions occurred in all cases, but these were not RIA positive. Fractions 1 and 3 occurred consistently in all life stages (except unfed larvae and unfed nymphs) (fig. 2). Increases in the concentration of fractions 1 and 3 occurred following larval engorgement and continued during molting. These changes paralleled the changes in concentration measured by RIA. However, the amounts of ECD detected, in ecdysone equivalents, were much higher than that detected by RIA. Thus, on day 2 post-dropping, the estimated total ECD concentration (fractions 1 and 3) was 0.384 ng. ECD concentrations declined as the larvae approached ecdysis and continued to decline in the young unfed nymphs. However, during and after nymphal engorgement, a tremendous surge of new ECD synthesis occurred and the ECD concentrations increased to more than 5 nanograms/tick. In addition, a new RIA positive fraction, fraction 2, appeared for the first time and increased during molting while the concentrations of the other fractions declined. All three fractions were represented in the adult stages (fig. 3). Fractions 2 and 3 were very abundant in emerging adults (day of emergence, day 0), but declined gradually as the unfed adults matured. ECD synthesis was found to occur during female tick feeding. The very great increase in concentration on day 7 was due almost entirely to a very great rise in the concentration of fraction 2.

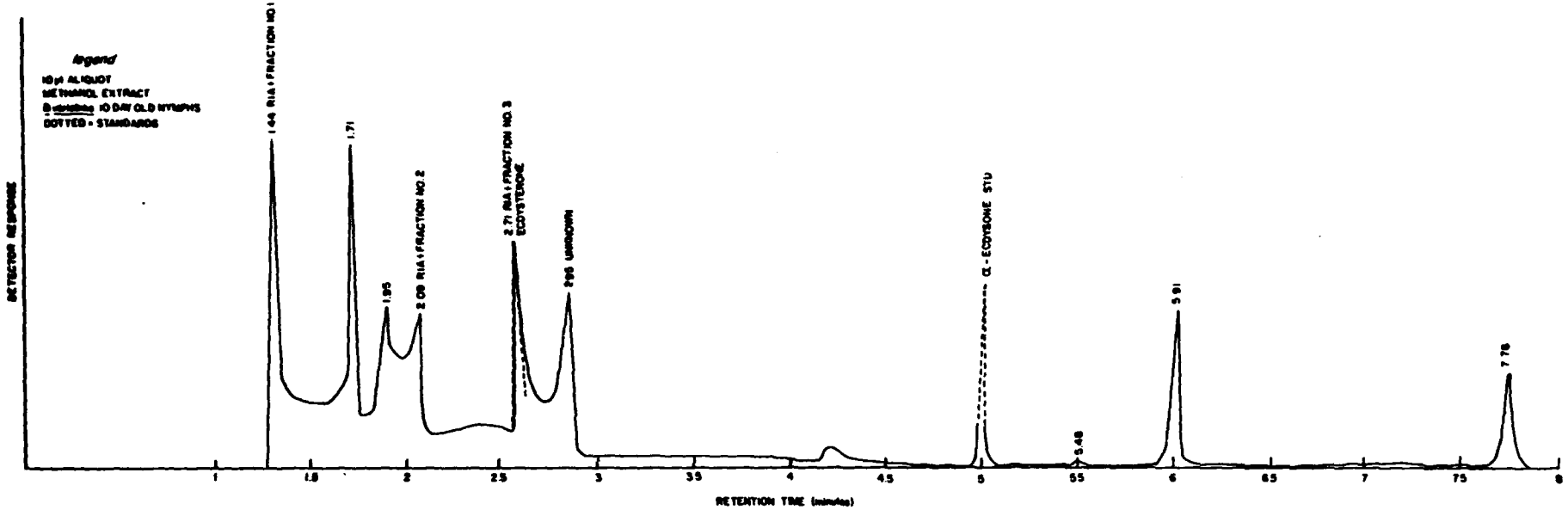

Figure 1. Representative HPLC chromatogram illustrating the retention times of the various fractions present in a sample of a text extract.

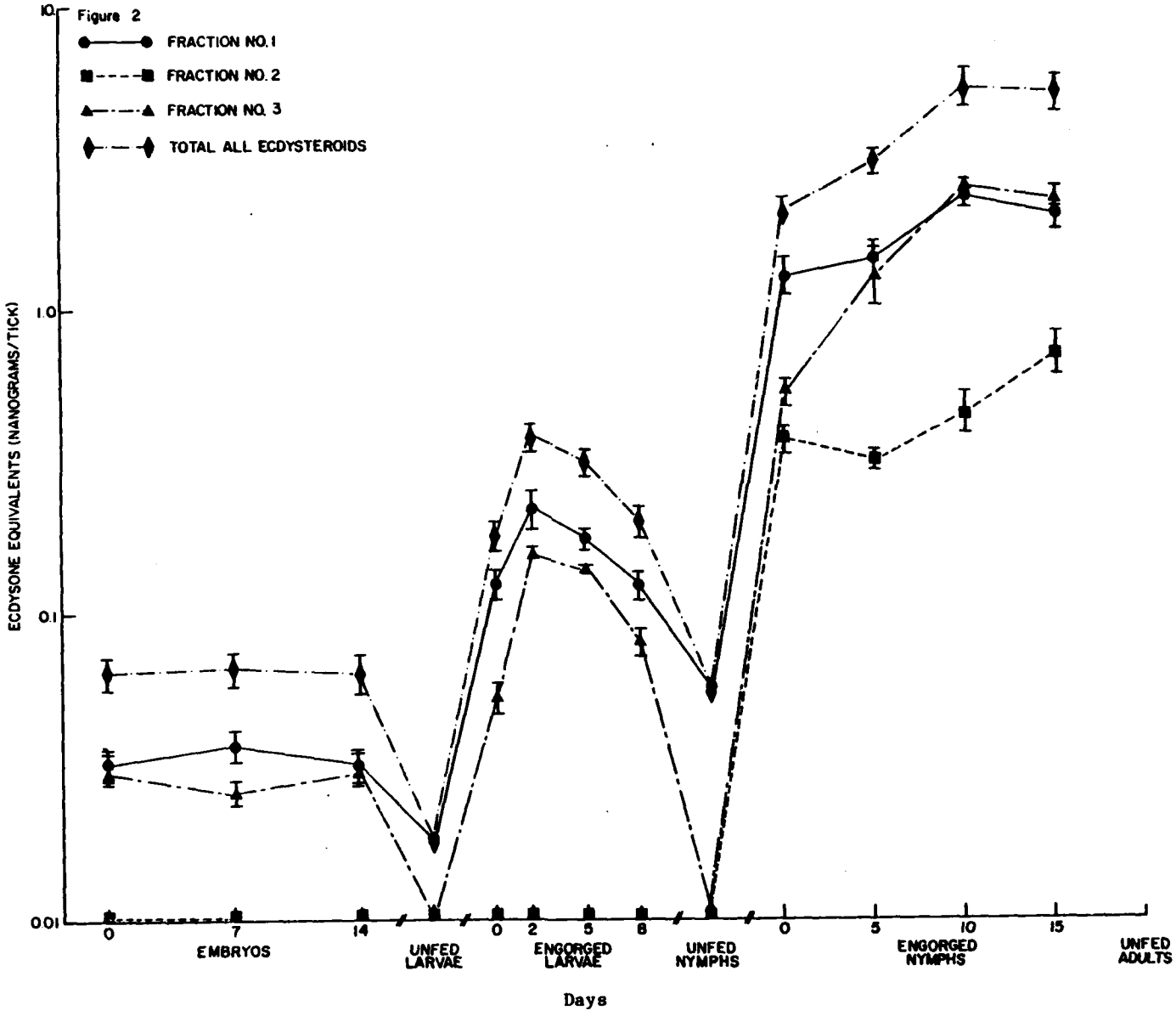

Figure 2. Concentrations (ecdysone equivalents) of different RIA positive fractions isolated by HPLC in different stages of immature D. variabils development. Values are means ± S.D.

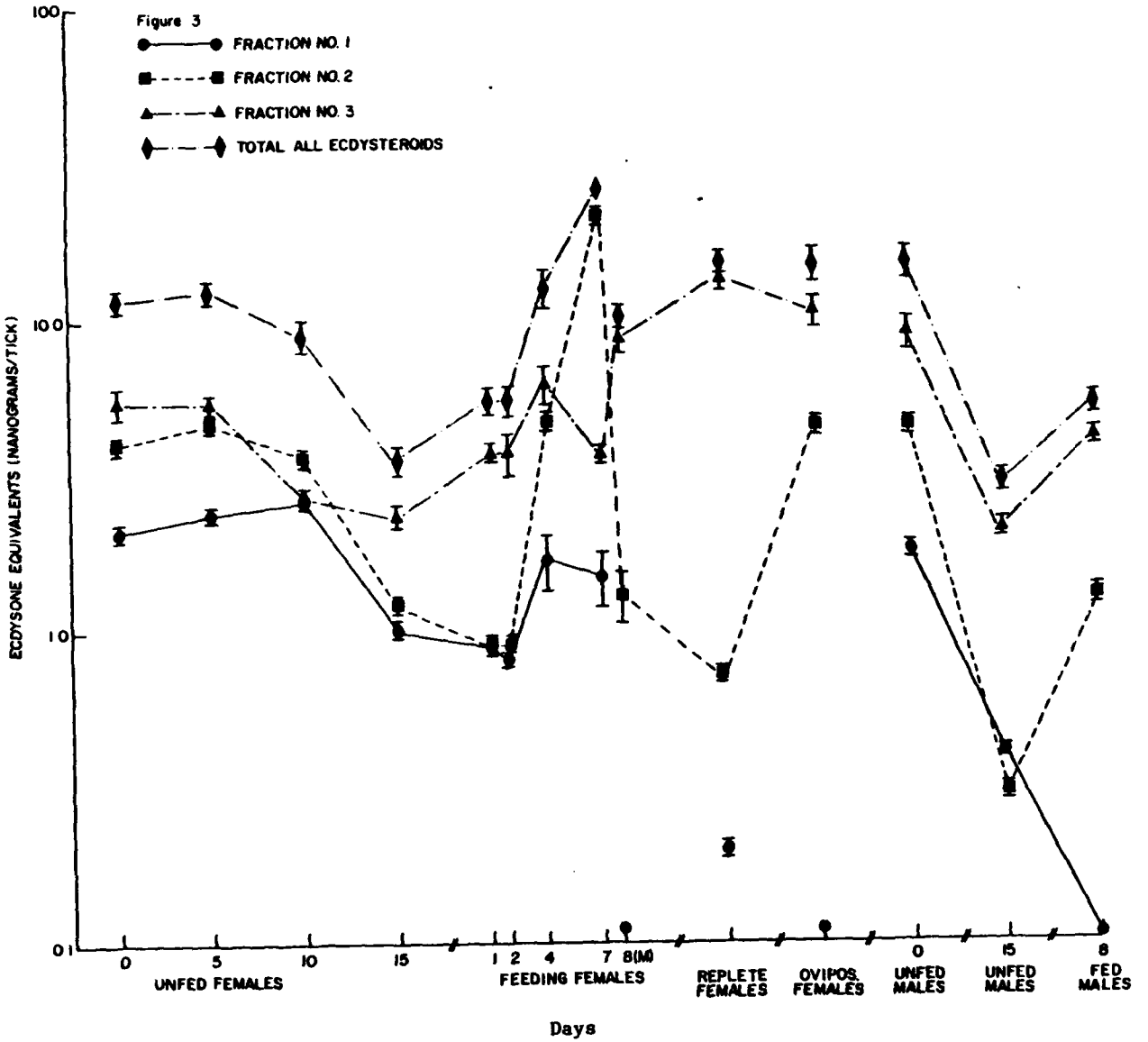

Figure 3. Concentrations (ecdysone equivalents) of different RIA positive fractions isolated by HPLC in different stages of adult D. variabilis activity. Values are means ± S. D.

Sex pheromone content. No sex pheromone, 2,6-dichlorophenol (2,6-DCP, was found in females on the day of emergence. DCP first appeared in females examined 5 days post-molting and increased to 2.6 and 1.1 ng/tick on days 10 and 15, respectively. Attached, feeding ticks were found to contain from 2.1 to 4.2 ng/tick, with little change in DCP content in relation to duration of attachment (table 2).

Ultrastructure. When examined with the transmission electron microscope, the secretory lobe cells of the pheromone glands of ecdysone treated unfed females (from 10 ng beta-ECD inoculated nymphs) exhibited extensive vesicular disruption and numerous free neutral lipid secretory droplets. Extensive vacuolation and droplet free areas were common (fig. 4). In contrast, the secretory lobe cells of the pheromone glands of unfed females controls (from 1% saline inoculated nymphs) did not exhibit disruption of the vesicles. All of the neutral lipid secretory droplets were observed to be bound within intact vesicles (fig. 5).

Discussion

Evidence of ecdysteroid presence was found during all periods in the life of D. variabilis, from early embryonic development to ovipositing females. The RIA values illustrate the changes in ecdysteroid content that occur during tick development and maturation. The RIA values should be considered as estimates. The most reliable estimates can be obtained only with the homologous antigens. Relative values may be determined for heterologous antigens when their identities are known and binding characteristics have been determined (Reum and Koolman, 1979). Ecdysteroid metabolites and other closely related steroids may also react (Borst and O'Connor, 1972) and, therefore, also contribute to the total estimate; however, their binding with the highly sensitive Horn I-1 antiserum is very slight (Reum and Koolman, 1979). Consequently, the RIA estimates reported in this study may substantially underestimate the total ecdysteroid content in these ticks. Nevertheless, the use of RIA remains a valuable tool for monitoring ecdysteroid activity because of its high selectivity; in addition, a considerable body of data based on RIA estimates is available for insects and ticks, providing interesting and useful comparisons.

Figure 4

Figure 5

Figure 4. Transmission electron micrograph of the foveal gland of an unfed D. variabilis female that emerged from nymph inoculated with 10 ng beta-ecdysone.

Figure 5. Transmission electron micrograph of the foveal gland from a D. variabilis treated as an engorged nymph with 1% saline.

The rise in ecdysteroid titers during developmental processes in D. variabilis, e.g., embryonating eggs, feeding and engorged immatures, appears to be consistent with the molting process in ticks as described by other authors (reviewed by Solomon et al. 1982). The peak ecdysteroid titer during nymphal molting occurred at 10 days post-feeding, or approximately 61.2% of the time course of development [according to Dees et al., in press, D. variabilis nymphs held at 27±1°C molt in 16.34 days]. This is only slightly less than the time of peak ecdysteroid occurrence in Amblyomma hebraeum Koch. Diehl et al. (1982) observed a sharp rise in ecdysteroid content in engorged A. hebraeum nymphs on day 23, or ca 67.7% of the time to ecdysis. The amount of ecdysteroid found by Diehl and his co-workers in A. hebraeum, during the first 16 days of nymphal molting, ca 1 ng or less, compares very closely with the amounts observed in D. variabilis nymphs. However, the surge in A. hebraeum ecdysteroid content to 14 ng/nymph at the time of peak activity is much higher than any of the ecdysteroid titers observed in D. variabilis. Estimates of ecdysteroid titers in A. hebraeum given by Delbecque et al. (1978), up to 200 pg/mg nymphal tick tissue, are also considerably higher than that observed in D. variabilis. No evidence of fluctuating ecdysteroid levels or multimodal peaks, such as that described by Delbecque et al. (1978) was found in D. variabilis; however, the 4 data points taken for molting larvae and nymphs may not have been sufficiently frequent to detect minor fluctuations.

Chromatography (HPLC) of the tick ecdysteroids reveals the presence of different steroids, at least 3 of which react in the radioimmunoassay with antiserum against insect ecdysone. One of the fractions, fraction 3, co-chromatographs with authentic beta-ecdysone (ecdysterone) and is almost certainly this molecule. This compound is present in almost every life stage or physiological state. It is absent only in the unfed larva and unfed nymph. The other 2 compounds, presumably ecdysteroids also, are considerably more polar than ecdysterone, but remain otherwise unidentified. Fraction 1 occurs in all life stages and physiological states, even the unfed larvae and nymphs. Most probably, it is a metabolite of ecdysone or ecdysterone, but the possibility of independent stable hormonal status for this compound cannot be excluded. Fraction 2, also of unknown identity,

appears only after nymphal engorgement, and increases in concentration thereafter. It increases enormously during adult feeding and, by attachment day 7, accounts for almost all of the very high ecdysteroid concentration observed in feeding females at that time. It also increases during male attachment and feeding. The late appearance of this second ecdysteroid and its increase during adult feeding, suggest a possible correlation with reproductive activity. The role of ecdystone in regulating insect vitellogenesis is well known (Hagedorn, 1974; Hagedorn, 1980). Evidence of a similar function in ticks is reviewed by Solomon et al. (1982), though the hormone responsible for initiating vitellogenic activity is believed to be ecdysterone.

Overall, HPLC gave much higher estimates of ecdysteroid content in the ticks than did RIA. This is not surprising, in view of the reduced reactivity of the antisera to heterologous antigens, as noted above. In the present study, none of the ecdysteroids detected was alpha-ecdysone, the homologous antigen. Thus, the heterologous ecdysteroid antigens observed may be expected to react to a much lesser degree when exposed to the alpha-ecdysone antiserum, and produce much lower values than the estimates obtained by HPLC.

Hormonal regulation of sex pheromone activity in ticks was suggested by Dees et al. (in press), who reported increased 2,6-DCP content, onset of pheromone gland secretion, and female sex attractant activity in unfed H. dromedarii following treatment with ecdysterone. Ecdysteroids may affect sex pheromone activity by mediating dopa-decarboxylase activity, and, in turn stimulate secretion of stored 2,6-DCP and concomittant DCP biosynthesis. Schlaeger et al. (1974) demonstrated ecdysone mediated stimulation of dopa-decarboxylase activity in mosquito ovaries. Cataecholamines, which occur in ticks (Binnington and Stone, 1977) and which stimulate tick salivary gland secretion (Kaufmann and Phillips, 1973), also appear to occur in the foveal glands (Sonenshine, unpublished). Thus, a correlation between ecdysteriod concentration and sex pheromone activity might be expected in D. variabilis. However, the results show little evidence of such a relationship. In the present study, concentrations of all 3 ecdysteroids increased during nymphal molting, when the foveal glands differentiate (Khalil et al.

1983). In unfed females, beta ecdysone remained elevated during the first 5 days post-emergence, when 2,6-DCP first appeared, but declined during the succeeding days when 2,6-DCP concentrations increased. The other ecdysteroids, fractions 1 and 2, declined between days 10 and 15, when the 2,6-DCP concentrations also declined. In feeding females, beta-ecdysone concentrations increased substantially during days 1 and 2 post-attachment, when release of pheromone occurs (Sonenshine et al. 1974) and new synthesis of 2,6-DCP commences (Sonenshine et al. 1977). In contrast, concentrations of the other ecdysteroids declined slightly during this same early attachment period.

The only direct evidence of the ability of the sex pheromone glands to respond to ecdysteroids is found in the effects of ecdysterone on foveal gland ultrastructure. The extensive vacuolation, vesicular disruption and release of lipid droplets following this treatment (fig. 6, 7) are features characteristic of the actively secreting gland. However, other studies with exogenous ecdysteroids in D. variabilis did not reveal any effect on sex pheromone activity; treated females did not contain more 2,6-DCP than controls, nor did they attract sexually excited males (Dees et al. in press).

Further study is needed to clarify the role of the different ecdysteroids in the physiology of ticks, and to differentiate the effects, if any, of the several different ecdysteroids which have been observed.

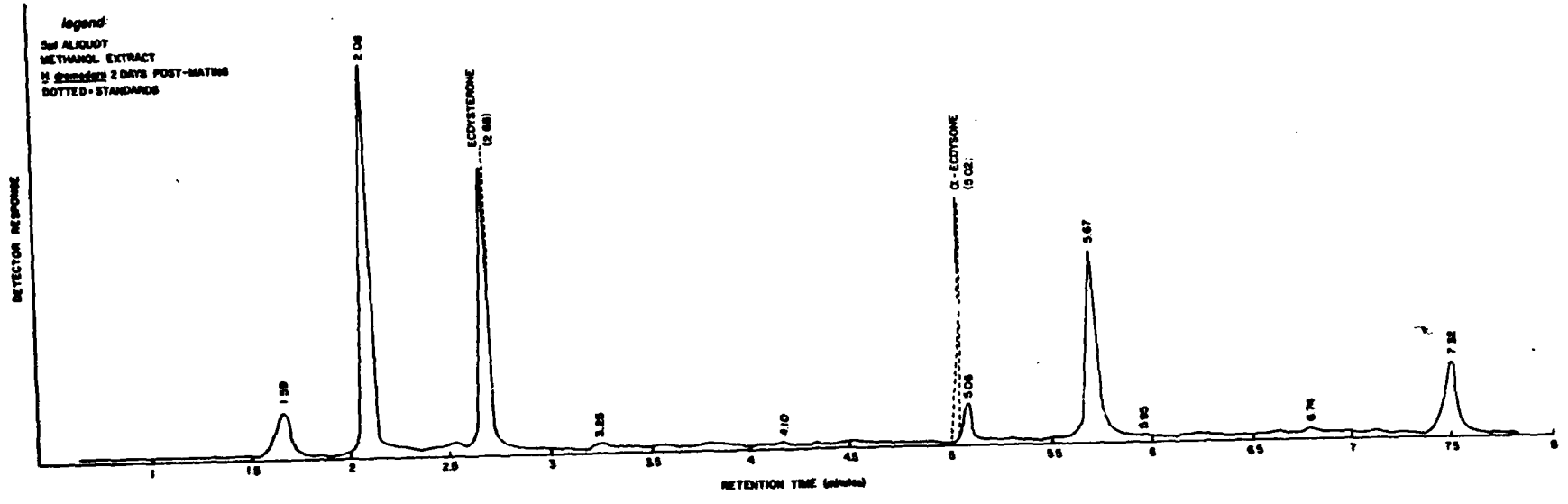

Figure 6. Representative HPLC chromatogram illustrating the retention times of the various fractions present in a sample of an extract of adult H. dromedarii.

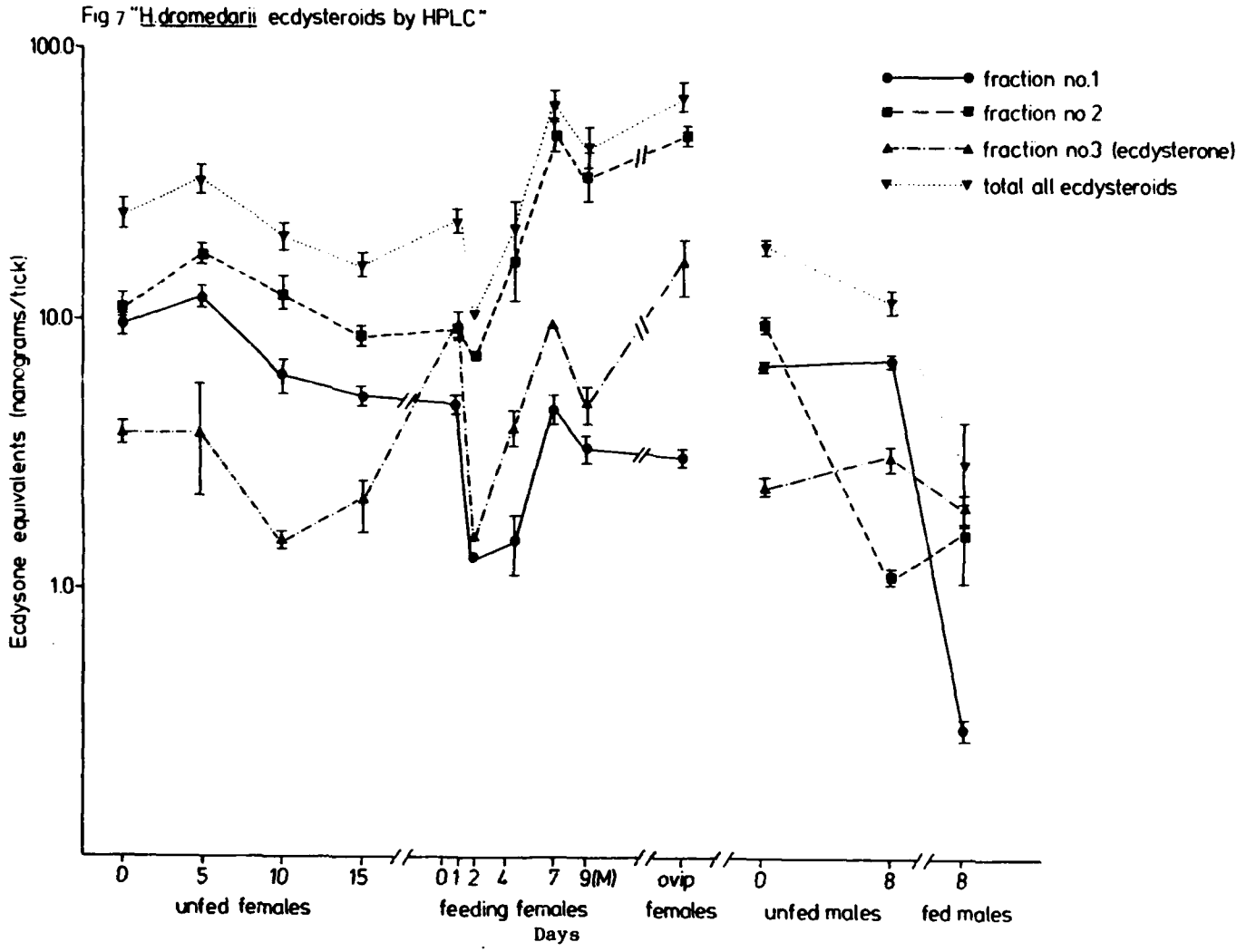

Figure 7. Concentrations (ecdysone equivalents) of different RIA positive fractions isolated by HPLC during different stages of adult H. dromedarii activity.

III. Ecdysteroids in the Camel Tick, Hyalomma dromedarii Koch During Different Periods of Adult Tick Development (Acari:Ixodidae) (ODU)

Introduction

This study was done to determine the types of ecdysteroids that occur in Hyalomma dromedarii and their role, if any, in sex pheromone activity. The rationale for the hypothesis that ecdysteroids exist in ticks, including H. dromedarii, was given in the preceeding (section 2) and by Dees et al. (in press). This study was limited to the adult stages. In this case, we were interested in determining whether the spectrum of ecdysteroids, including ecdysone and ecdysterone, found in other species also occurred in H. dromedarii. We were also interested in measuring the concentrations of the different ecdysteroids which proved to be positive in our radioimmunoassay and comparing these concentrations with important physiologicl events, e.g. feeding, onset of sex pheromone activity, mating and repletion. Knowledge of the specific hormones, hormone metabolites, and changes in their concentrations may be important in evaluating hormone-pheromone relationships.

Materials and Methods

Ticks. The camel tick, H. dromedarii, was colonized and reared with specimens from the Medical Zoology Department, U. S. NAMRU-3, Cairo, Egypt (HH#59,723; U. S. APHIS permit #9433). Specimens were fed on laboratory on rabbits [Oryctalagus cuniculus]. Ticks were held in an Aminco Climate Lab environmental chamber at 27±0.5°C and 90± 2% RH during their non-parasitic periods.

Tick extracts. The materials used for radioimmunoassay (RIA) are listed in Table 3. Extracts of each population were prepared by homogenizing the tick material with a tenBrock glass homogenizer. (American Scientific Products, Columbia, MD) in methanol and water (65:35, v/v). The mixture was freeze-thawed, centrifuged, and washed 2 X with this same solvent. The precipitate was discarded, the supernatant evaporated, and the dried sediment re-extracted 3 X with 100% reagent grade methanol (J. T. Baker Co.,

Table 3. Ecdysteroids concentrations in Hyalomma dromedarii during different periods of adult activity as determined by RIA[1,2]

Life Stage	Days Post-eclosion/ Feeding/mating	ng/tick ± S.D.	ng/mg ± S.D.
unfed females	0[3]	1.34 ± 0.21	0.06 ± 0.01
"	5	2.41 ± 0.60	0.16 ± 0.04
"	10	0.85 ± 0.27	0.06 ± 0.01
"	15	1.63 ± 0.56	0.11 ± 0.03
unfed males	0	0.87 ± 0.39	0.05 ± 0.02
"	15	0.48 ± 0.14	0.03 ± 0.01
feeding females (virgin)	1	0.44 ± 0.05	0.031 ± 0.004
"	2	0.55 ± 0.16	0.033 ± 0.009
"	4	1.55 ± 0.56	0.046 ± 0.017
"	7	1.45 ± 0.44	0.015 ± 0.005
feeding females 2 days post-mating	9[4]	6.78 ± 1.37	0.020 ± 0.004
replete	11	18.85 ± 7.38	0.027 ± 0.010
fed male	11	0.51 ± 0.20	0.025 ± 0.009

1 Data for unfed females day 0 and unfed males are from Dees et al. (in press) with permission of the editor.

2 Ten (10) individuals assayed in all instances unless specified otherwise.

3 Twenty (20) individuals assayed in this instance.

4 Five (5) individuals assayed in this instance.

Phillipsburg, NJ) with vigorous shaking. The methanol extract was centrifuged to remove extraneous material, dried, re-extracted 4 X with 50% methanol/benzene, again with 25% methanol/benzene, and finally, reconstituted in 100% ethanol. The final extract was stored (-20°C.) until needed for assay. Samples of these extracts were also assayed by High Performance Liquid Chromatography, as described below. The putative ecdysteroids were collected, and the fractions were assayed by RIA.

The tick material used to determine the presence of sex pheromone, 2,6-dichlorophenol (2,6-DCP) is listed in table 4. The tick material was extracted in double-distilled hexane Omni- Solv (Krackler Chemical Co., Albany, NY) as described previously (Sonenshine et al. 1977; Sonenshine et al. 1982).

Radioimmunoassay (RIA). Radioimmunoassay was used to determine total ecdysteroid concentrations and confirm the identity of compounds isolated by chromatography as ecdysteroids. The Horn I-1 anti-ecdysone anti-serum used in these tests was obtained (as a lyophilized powder) from Dr. J. D. O'Connor, University of California, Los Angeles. It was reconstituted in distilled water and stored frozen (-65 C) until needed. Aliquots of the tick extracts (methanol) described above were dried under nitrogen and reconstituted in 100 ul borate buffer. These were mixed with 50 ul aliquots (also in borate buffer) of tritiated alpha-ecdysone (16,000 DPM) (New England Nuclear Corp., Boston, MA, 80 Ci/mmol) and 50 ul of antiserum. The mixture was incubated for 24 hours at room temperature. After cooling (4 C), 200 ul of a saturated ammonium sulfate was added to each tube. The proteins were allowed to precipitate overnight and the solution was vortexed and centrifuged at 2500 rpm. After centrifugation, the supernatant was removed, the precipitate resuspended in fresh borate buffer:SAS solution (1:1), and the mixture centrifuged again. The supernatant was removed and the precipitate dispersed in 25 ul 100% ethanol plus 600 ul RIA Fluor (New England Nuclear Corp., Boston, MA) in minivials. Duplicate samples were prepared for each extract and each dilution of that extract. Standards were prepared in a similar manner using known quantities ranging from 25 pg/ul to 1000 pg/ul of authentic alpha-ecdysone (Sigma Chemical Co., St. Louis, MO). Radioassay was done with a Beckman Model LS 250 Liquid scintillation count-

Table 4. Changes in the concentration of sex pheromone, 2, 6-dichlorophenol in unfed, partially fed, replete and ovipositioning?

Hyalomma dromedarii

A. Unfed Days Post-eclosion	Sex	No. in Sample	ng DCP/ Tick
0	Female	51	0.00
5	"	100	1.08
10	"	109	17.29
15	"	140	16.32
15	Males	161	0.00

B. Part-Fed and Replete Days post-attachment	Sex	No. in Sample	ng DCP/ Tick
1	Females	74	3.90
2	"	67	4.04
4	"	61	10.86
7	"	50	6.71
9*	"	25	15.65
10**	"	25	13.2

* 2 days post-mating

** Replete, usually day 11

er. Counting efficiency after quench correction (external standard method) was 29%.

The purity of the ^{3}H alpha-ecdysone was confirmed by thin-layer chromatography (TLC) before use in the various tests (see below).

Chromatography (HPLC). The chemical characteristics of the ecdysteroids in the tick extracts were determined by TLC and High Pressure Liquid Chromatography (HPLC). TLC was done on Whatman K5WF thin layer silica gel plates (Whatman, Inc., Clifton, NJ) in chloroform: methanol (4:1, v/v). HPLC was done with a Waters System comprising Model 6000 and M45 pumps, Model 720 system controller, 730 Data Module, Model u6K injector, and a model 440 UV absorbance detector (Waters, Inc., Milford, MA). The column was a model RCM, 8 mm I. D, 10 uM MicroBondapax C-18 column. The solvent system was methanol:water:acetic acid (65:35:1, v/v) and the flow rate was 1.5 ml/min. In later studies, the column was a 10 uM Radial Pak C-18 column, a 5 um x 10 cm. was also used. The same solvent system was used but the radio of the solvents was reduced to 45:55:1, and the flow rate was reduced to 1.0 ml/min. The ecdysteroids in the tick ex- tracts were identified by comparison of their retention times with authentic insect ecdysones and by radioimunoassay of the specific isolates. Samples selected for analysis consisted of aliquots of 50 - 100 ul of the original extract of the different life stages assayed by RIA (table 3), and recon- stituted in reagent grade methanol; 10 ul aliquots were injected into the HPLC.

Sex pheromone content (GLC). To determine the amounts of sex pheromone in the ticks, hexane extracts of tick material were passed through pre-cleaned Sep Paks (Waters and Associates, Inc., Milford, MA) containing Florasil to separate the 2,6-dichlorophenol from other organic materials. The Sep Paks were rinsed with fresh hexane (3 X to release residual non-polar molecules, then rinsed with a mixture of 1:1 diethyl ether:petroleum ether to release the phenol. Recovery with this technique is approximately 100%, as confirmed with tests with known standards. Aliquots of the final extract were injected into a Shimadzu GC-6A gas chromatograph (Shimadzu, Inc., Rockville, MD) using Hamilton Model 701-RN microliter syringes with Chaney adapter and 26 S gauge needles. The gas chromatograph was equipped

with a constant-current electron capture (EC) detector with a ^{63}Ni foil to detect halogenated compounds. Dector and injection port temperatures were 250°C, the column was 200°C. and the flow rate for the carrier gas (N2) was 75 ml/min. The column was a 0.6 cm O.D. x 56 cm long curved glass tube packed with Tenax 80/100 absorbent. Recordings and quantification of compounds were made with a Shimadzu C-RIA Chromatopac Multiprocessor (Shimadzu, Inc., Rockville, MD) connected to the gas chromatograph.

Identification of 2,6-dichlorophenol in the extracts was done by comparing sample compound and authentic 2,6-dichlorophenol retention times, and by coinjection of the authentic standard and observing that it co-chromatographed with the sample compound.

Results

Radioimmunoassay. Ecdysteroids were present in both males and females, in unfed individuals, and at various times during feeding, repletion, and oviposition. When extracts of these adult stages were assayed by RIA, ecdysteroid titers ranging from as little as 0.44 ng to as much as 18.85 ng/tick were observed (table 3). In unfed females, considerable fluctuation in ecdysteroid titers occurred, but no consistent trend was detected during the 2 week period following emergence from the nymphal molt. Ecdysteroid titers rose significantly during the first few days of feeding by virgin females ($t = 4.89$, $p < 0.01$). However, a very sharp increase, more than 13 fold, occurred after mating and the period of rapid engorgement that follows. Ecdysteroid titers in unfed males were slightly less than in unfed females at comparable periods, and declined during the 2 week period following emergence from the nymphal molt.

Chromatography (HPLC). Although as many as 6 different major fractions could be detected when tick extracts were assayed by HPLC (on C-18 reversed phase columns), only 3 fractions proved RIA positive when assayed with the Horn I-1 antiecdysone antiserum. Figure 6 shows the relative abundance of these RIA positive steroids, or ecdysteroids, during the various stages of adult life in H. dromedarii. Fraction 3 was identified as beta-ecdysone (ecdysterone) since it co-chromatographed the authentic standard compound.

Fractions 1 and 2 are more polar, and may be polar metabolites of ecdysterone. One and occasionally two other peaks more polar than ecdysone also appeared, but neither was RIA-positive. Although no unequivocal evidence of ecdysone (alpha-ecdysone) was detected, a very small peak occasionally appeared within 0.03 to 0.1 min of the time of appearance of the alpha ecdysone standard, and is probably this compound. Often, it could not be detected at all. In 4 extracts, (1) unfed females 15 days post-eclosion, (2) females attached 2 days (part fed), (3) females attached 4 days part-fed) and (4) females attached 9 days (2 days post-mating); estimates of the amounts of this putative alpha-ecdysone were 0.02, 0.11, 0.06, and 0.03 ng/tick. In addition, two other relatively apolar compounds were found, but did not react in the RIA when assayed.

HPLC detected much greater quantities of the several ecdysteroids than RIA, as much as 50 fold greater quantities in some instances, even though both types of assays were done with the same extracts. In unfed females, the ecdysterone concentration diminished only slightly following eclosion, but was elevated (on most days) during attachment and feeding. Following an initial rise, the ecdysterone concentrations fluctuated from day to day, but rose very sharply with the commencement of oviposition. The concentration of fraction 1 diminished gradually during female maturation and the early period of female feeding, rising slightly during the later period of feeding, repletion and oviposition. Fraction 2 was generally the most abundant of the 3 ecdysteroids. It also exhibited the most dramatic increase during feeding, to as much as 48 ng/tick during the later stages of feeding, repletion and subsequent oviposition. Marked differences in the concentrations of the other fractions noted above were also observed, but these differences in concentration are not reported here since the compounds were not RIA positive.

Data on the occurrence and relative concentration of 2,6-dichlorophenol (2,6-DCP) is still being collected. Table 4 summarizes our findings to date for H. dromedarii. At present, our evidence reveals the absence of any detectable sex attractant pheromone in newly emerged females; 2,6-DCP was evident on day 5 post-emergence, and increased several fold by day 15. The amounts of 2,6-DCP observed in feeding virgin H. dromedarii females attached

1 and 2 days was 3.70 and 4.04 ng/tick, respectively. Collections to determine the amounts present in females attached 4 or 7 days, mated females and replete females are still being accumulated. These samples will be assayed as soon as sufficient specimens have been accumulated to provide meaningful sample sizes.

Discussion

These studies confirm the previous findings of ecdysteroids in H. dromedarii by Dees et al. (in press). These authors noted slight changes in the total ecdysteroid content between newly emerged and 2 week old adults, and between unfed and feeding females. The results of the present study confirm these findings and, in addition, demonstrate that ecdysteroids are present during all phases of adult physiological activity (except the ovipositing female, which was not investigated).

The changes in ecdysteroid content during different periods of adult activity suggest possible correlation with important physiological events. Both RIA and HPLC estimates indicate a very sharp rise in ecdysteroid content in female ticks feeding for 7 days, as well as in mated and replete females. When the quantities of the several RIA positive fractions isolated by HPLC are compared, it is evident that the major reason for this sharp increase at these points in time is due to fraction 2, which increased to between 34 to 48 ng/tick. Increases in the amounts of fraction 3, ecdysterone, also occurred, especially during the final period of feeding between mating and repletion (rapid engorgement phase).

The 3 RIA positive ecdysteroid fractions found in the different physiological states of H. dromedarii adults are very similar to that observed by Dees et al. (unpublished) in D. variabilis. Ecdysterone (= fraction 3) which is present in H. dromedarii, was also found by Dees et al. in D. variabilis. The differences in retention time were 0.06 min for fraction 1 and 0.01 for fraction 2, respectively, when samples from H. dromedarii and D. variabilis were analyzed sequentially using identical conditions; no differences could be discerned when samples from the 2 species were combined and injected together. These findings suggest that fractions 1 and 2 are very similar or identical molecules in the two species. Identification of the 2 fractions can only be done by further chemical study, especially mass fragmentography.

Ecdysone also appears to be present in H. dromedarii, but in low concentrations, often below the level of detectability by HPLC.

Comparison of the ecdysteroid in H. dromedarii and Amblyomma hebraeum reveals some interesting similarities and noteworthy differences. Delbecque et al. (1978) reported an estimated "25 ug or more of ecdysteroid-like material as determined by RIA" from engorged A. hebraeum nymphs 17 days post post-dropping, or ca. 16.7 ng/tick. This value is considerably higher than our estimates for day of emergence adults (1.34 ng/female, 0.87 ng/male), but no data for nymphal H. dromedarii was collected and direct comparisons are impossible. In a later study, Diehl et al. (1982) reported peak values of up to 14 ng/tick in engorged nymphs 23 days post-dropping, but only ca. 0.5 ng/tick in freshly molted females. Thus, our values for H. dromedarii females on the day of emergence, 1.34 ng/tick, are comparable to that reported for the same period in A. hebraeum, though ca. 2.5 times greater. Diehl et al. (1982) also reported substantial quantities of alpha-ecdysone in A. hebraeum, though the amounts found were always much less than that of ecdysterone, in some cases as little as 5.7% of the ecdysterone concentration.

Bouvier et al. (1982) studied ecdysone metabolism in Ornithodoros moubata (Murray, 1877; sensu Walton, 1962) using ^{3}H labelled authentic ecdysone. Conversion to ecdysterone was confirmed by demonstration of radiolabelled ecdysterone in ticks injected with ^{3}H ecdysone. In addition, other radiolabelled compounds were produced. Two fractions were more polar than ecdysterone. One of these, identified as 20, 26 diOH-ecdysone, exhibits chromatographic separation profiles similar to that of fraction 2 in H. dromedarii; it appears slightly earlier than ecdysterone in both species. Another fraction, of unknown identity, appears much earlier, and resembles fraction No. 1 in H. dromedarii. In both species, there were several fractions more polar than ecdysterone (i.e., both species exhibit fractions more polar than ecdysterone). Fraction 1 was found to be pH sensitive in O. moubata and its retention time is extremely dependent upon solvent pH, a finding which may explain the difficulties we obtained in detecting and/or collecting fraction No. 1 in H. dromedarii. In addition, changes in solvent pH resulted in subdivision of Bouvier et al.'s fraction 1

into several peaks, which they interpreted as indicating that this fraction contained an ionizable compound or compounds; the several peaks presumably represent different ionizing states. A similar ionizable molecule in H. dromedarii (and D. variabilis) could explain the occurrence of 3 or 4 peaks appearing earlier than ecdysterone. Other similarities between H. dromedarii, D. variabilis and O. moubata also occur. All 3 species exhibit relatively apolar fractions, of unknown identity. All 3 species appear to lack makisterone, and 2-deoxy-ecdysone, ecdysteroids common in insects.

The occurrence of ecdysteroids has now been confirmed in 7 species of ticks, including O. moubata (Bouvier et al. 1982), A. variegatum (Ellis and Obenchain, cited by Solomon et al. 1982), A. hebraeum (Delbecque et al. 1978), Ornithodoros porcinus (Mango and Obenchain, cited by Solomon et al. 1982), Rhipicephalus appendiculatus (Whitehead et al., cited by Solomon et al. 1982), H. dromedarii (Dees et al. 1982), and D. variabilis. Clearly, the occurrence of these compounds is widespread in ticks, perhaps universal. Ecdysterone was found in O. moubata, A. hebraeum, and R. appendiculatus (reviewed by Solomon et al. 1982), D. variabilis and H. dromedarii. Ecdysone was reported in O. moubata (Germond et al. 1982), A. hebraeum (Delbecque et al. 1978) and R. appendiculatus (Solomon et al. 1982); it may also occur in D. variabilis and H. dromedarii, but at concentrations too low to insure detection.

Further studies are needed to determine the role of the various ecdysteroids in the different physiological activities of ticks. It is especially important to determine whether one molecule, e.g., ecdysterone, is the active hormone which regulates the various events, or whether different compounds regulate specific processes, independent of one another.

IV. Studies on the Origin (Precursors) and Fate of Ecdysteroids in Hyalomma Dromedarri) (ODU)

Introduction

These studies were undertaken to determine the precursor(s) of ecdysteroid biosynthesis and metabolic byproducts, if any, in ticks. In addition, we were interested in determining whether ecdysteroid hormones are transferred transovarially from one generation to the next, or must by synthesized de novo in each generation.

Materials and Methods

Engorged Hyalomma dromedarii nymphs were collected on the day of drop-off and each nymph was injected with 2 ul of a solution containing 0.50 uCi of ^{14}C cholesterol, labelled on the c-26 and C-4 position (50 uCi/mmol and 57 uCi/mmol, respectively) (New England Nuclear Corp., Boston, MA). The cholesterol was dissolved in a solution of 1% citric acid and 5% propylene glycol in sterile Shen's solution, in which the material dissolved readily. Following the injections, the treated nymphs were held at 27±1°C and 92 ±2% RH in the Aminco Aire Climate Lab until they molted. Two weeks post-molting, 10 males and 10 females from the treated population were weighed (avg. wt. of males 18.17 mg; of females, 16.30 mg) and then homogenized separately with a Ten Broeck glass homogenizer (American Scientific Products, Columbia, MD) in methanol and water (65:35, v/v). The mixture was freeze-thawed, centrifuged, and washed 2X with this same solvent. The precipitate was discarded, the supernatant evaporated, and the dried sediment re-extracted 3X with 100% reagent-grade methanol (J. T. Baker Co., Phillipsburg, NJ) with vigorous shaking. The methanol extract was centrifuged to remove extraneous material, dried, re-extracted 4X with 50% methanol/benzene, again with 25% methanol/benzene, and finally reconstituted in 1 ml. of 100% ethanol. A more detailed description of the extract procedures was given in the preceding sections (sections II and III).

Aliquots of the male and female extracts described above were analyzed by High Pressure Liquid Chromatography, using a Waters Associates HPLC system (Milford, MA), and all the steroid peaks were collected for radioassay. HPLC parameters were as follows: solvent system, 45:55 methanol/water; flow rate 1.5 ml/min; column was a 5 mm, 10 um C-18 cartridge (waters Assoc., Milford, MA). Collections were made based upon previous studies which demonstrated the existence of 3 radioimmunoassay positive peaks, including ecdysterone. Collection No. 1 consisted of the combination of the first 2 peaks, which were more polar than ecdysterone. Collection No. 2 represented ecdysterone, and was collected separately from all the others. Collection No. 3 consisted of the combined collection of all the remaining peaks which appeared after ecdysterone.

For radioassay, the collections were transferred to liquid scintillation cocktail vials each containing 10 ml of the scintillation fluid, Dimilune (Packard Inst. CO., Downer's Grove, IL). Radioassay was done with a Beckman LS 250 Liquid Scintillation Counter (Beckman Inst. Co., Fullerton, CA), with corrections for quenching.

To test for transovarial transmission of ecdysteroids, 40 unfed D. variabilis females were inoculated (in the posterior abdominal body region) with 1 uCi/tick of ^{3}H alpha-ecdysone in ul of Shen's solution (80 mCi/mmol) (New England Nuclear Corp., Boston, MA) using a 30 gauge needle and 50 ul Hamilton syringe. The treated ticks were allowed to feed, copulate, and oviposit after engorgement. Egg masses were obtained from 9 surviving females. Following hatching, larvae were collected from all egg batches and divided into 3 groups of 150 larvae each. The larvae were solubilized with Soluene (Packard Inst. Co., Downer's Grove, IL) in liquid scintillation cocktail vials. The scintillation cocktail Dimilune was added to the dissolved material and transferred to the liquid scintillation counter for analysis. Radioassay for ^{3}H alpha-ecdysone was done as described above. The efficiency of the LSC For tritium was estimated at 42%. Predetermined quench curves were used to correct for quenching.

Autoradiographs were done with frozen sections of gonads, synganglia, and foveal glands of males and females that emerged from ^{14}C cholesterol

treated ticks. The tissues were quick frozen, sectioned in a cryostat, and fixed to slides. The dried slides were dipped in warm (40°C) Kodak NTB-3 nuclear emulsion and held for 10 days at 4°C. Following exposure of the emulsion, the slides were developed and stained with aqueous haematoxylin and eosin.

Results

Synthesis of ecdysterone from cholesterol. The fraction containing ecdysterone, Collection No. 2, demonstrated highly significant radioactivity, 892.8 cpm and 901.3 cpm over background, for female and male ticks, respectively. Much smaller amounts, 71 and 92 cpm over background, were found in the combined collection of peaks 1 and 2 (Collection No. 1), containing unknown RIA positive ecdysteroids. Substantial radioactivity was found in the combined fractions of all of the remaining compounds, Collection No. 3, containing non-RIA reactive compounds of unknown identity, 602.9 cpm for female ticks and 1474 cpm for male ticks, respectively.

Microscopic examination of the autoradiographs revealed substantial concentrations of silver grains over the testis of a male that emerged from the ^{14}C cholesterol treated nymphs. Some evidence of accumulation of grains over the reproductive tissues from a female was also found, but most of the ovary was lost in the process of specimen preparation. Significant accumulations of silver grains were also found over the cortical zone of the synganglia of both sexes and the foveal glands of a female. No evidence of silver grain accumulation was seen over epidermis or muscle fragments examined in this study. Additional observations are needed before precise counts can be determined for the different levels of ^{14}C accumulation over background.

Transovarial transmission of ecdysone. Radioassay of the larval digests for tritium revealed an average count of only 1.78 cpm/larva, or 2.6×10^{-6} uCi/larva. With a specific activity of 80 uCi/mmol, this represents less than 0.015 picograms per larva.

Discussion

In general, insects and acarines cannot synthesize sterols de novo from simple molecules. Rather, they must obtain these molecules in their diet. Ticks depend upon host blood and other fluids for their sterol source. Much of the cholesterol acquired in their blood meal is deposited, apparently unchanged, on the body surface as part of the lipid coat.

The ability of insects to convert sterols into ecdysteroids has been reported by several workers, with percentage incorporation ranging from 0.015 to 0.12% (Rees et al. 1980, review; Smith et al. 1980, review; Riddiford and Truman, 1978). In several species, conversions of radioactive cholesterol into ecdysone occurred in the prothoracic glands; in others, ovarian biosynthesis was demonstrated (Rees et al. 1980). Subsequent conversion to 20-hydroxecdysone occurs in a variety of tissues, but especially in fat body. Indeed, most of the alpha-ecdysone is hydroxylated to ecdysterone (=beta-ecdysone), which is many fold more active than alpha-ecdysone [Riddiford and Truman noted that beta-ecdysone is effective at about 1×10^{-7} M in simulating morphogenetic effects. When we injected 10 ng/tick into ticks in various studies reported in this and previous reports, we were injecting ca. 4.8×10^{-5}M].

Hypothetical schemes for biosynthetic pathways have been proposed for the synthesis of ecdysteroids (Riddiford and Truman, 1978; Rees et al. 1980). A common intermediate is 22-deOxyecdysone, a molecule very similar to ecdysone, but with only 4 hydroxyl groups. Catabolism of alpha and beta ecdysones leads to other, inactive polyhydroxylated molecules.

The conversion of labelled cholesterol to beta-ecdysone by H. dromedarii confirms the general similarity of the tick biosynthetic system to that of insects. Further research will be needed to determine whether the same intermediates or breakdown products are involved and the overall efficiency of the system.

V. Effects of Ecdysteroids and Juvenile Hormone Analogues on Development and Sex Pheromone Activity in Hyalomma Dromedarii (ODU)

Introduction

Treatment of insects and ticks with exogenous sources of hormones or hormone analogues is a widely used method for evaluating the role of such compounds in these arthropods (Solomon et al. 1982, review). In the case of H. dromedarii, evidence of ecdysterone exitation of sex pheromone activity was described by Dees et al. (in press). Little else is known about the effect of ecdysteroids on this species, and virtually nothing is known regarding the effects of juvenoids. Evidence of juvenile hormone (JH) activity in ticks was described by Pound and Oliver (1979) but this finding was for an argasid tick, Ornithodoros parkeri.

This brief report describes studies with slow release methods used to administer ecdysteroids and juvenoids in uniform amounts over extended periods of time. These included Methoprene, an extremely potent JH analogue high effectively against dipterous insect immatures (but little else), beta ecdysone, and an analogue of ecdysteroids, BSEA-28. The latter was synthesized by Thompson et al. (1971). These materials were incorporated into plastic tubing for implantation into rabbits to test their activity against H. dromedarii. This procedure was developed solely for the purpose of enhancing uniform delivery of the test compounds which would be taken up by the ticks in their blood meal. It represents a basic research investigation; implications regarding the efficacy of these materials and the method of delivery for control of this species are premature at this time. In addition to administration of the test material systemically, the effects of hormone administration were also tested by direct application to the ticks; the effects of JH, alone or in combination with ecdysterone, is also described in this study.

Materials and Methods

Methoprene (technical 91.9%) (Zoecon Corp.), beta-ecdysone (Sigma Chemical Co., St. Louis, MO), and an ecdysterone analogue, BSEA-28, were

obtained as active ingredients incorporated into plastic tubing (polycaprolactone with the ends sealed with silastic plugs) from personnel of the U.S. Department of Agriculture, Beltsville, MD (Dr. H. Jaffe). Methoprene was suspended in propylene glycol, while ecdysterone and BSEA-28 were suspended in ethyl oleate. The amounts (in mg.) administered with each treatment are given in Table 5. The plastic tubing strips in which these materials were incorporated were 40 mm long by 2.54 mm. O. D., with a wall thickness of 0.19 mm. The strips were implanted under the skin of rabbits, Oryctalagus cuniculi, 3.0 to 3.5 kg body weight previously tranquilized with Acepromazine (source). Both male and female animals were used. The implants were located in the shoulder area of the animal's back, in a shaved sector previously cleansed with 70% ethanol and hydrogen peroxide. Following implantation, the wounds were sutured using surgical suture needles and sterile technique, and the sutures left in place until the wounds had healed. Tubes with propylene glycol, but no active ingredients, were sutured into another rabbit and served as a control. Another rabbit, with no implant, served as an untreated control.

Following surgical implantation, suturing and recovery of the treated rabbits, the animals were held for observation and monitored for evidence of infection for 48 hours. Subsequently, the animals were shaved, plastic feeding capsules installed over stockinet, and the rabbits infested with ca. 300-400 H. dromedarii larvae. The rabbits were examined regularly for evidence of feeding and development of the ticks. Following the drop of the engorged nymphs, usually on the 20-22nd day after infestation, the engorged nymphs from each rabbit were counted and weighed using a Sartorius semi-microbalance (Brinkman Inst. Co., Westbury, NY).

In another experiment, H. dromedarii nymphs were treated with hormones on the day of drop off after engorging on rabbits. One group of nymphs, ca. 200 individuals, Group I, received inoculations of ecdysterone, 10 ng/tick, in sterile Shen's solution, administered in 2 ul aliquots with a Hamilton syringe and 30 gauge needle into the posterior body cavity. A second group, Group II, also ca. 200 nymphs, was treated as in Group I. However, after the introduction of the ecdysterone, these nymphs were affixed to double Scotch tape and treated with a solution of JH I dissolved in DMSO:Acetone, 10 ug/tick. The solution was administered in 2 ul aliquots with a Finpipette and disposable tip. Controls were treated with Shen's solution only.

Table 5. Effect of presence of ecdysteroid or IGR implanted into host rabbits on engorgement weight and molting in Hyalomma dromedarii

Type of Comp. Implant	Wt. Mater. (mg.)	Wt. of eng. nymphs No.	Wt(mg.)		No. Fed	No. Live	MOLTING Molting Dead	Duration (Days)	
Methoprene	41.9	48	20.92	4.08	72	71	1	22.19	1.76
"	81.3	45	32.98	4.65	102	101	1	22.97	1.89
Ecdysterone	47.5	87	26.40	4.87	199	193	6	20.39	2.41
BSEA-28	47.6	6	21.93	4.69	6	6	0	----*	
"	50.7	25	13.53 ±	4.79	75	73	2	15.43 ±	3.78
Propylene glycol	37.5	30	21.66 ±	4.67	74	72	2	21.35 ±	2.06
untreated control	----	24	20.53 ±	3.94	66	66	0	20.34 ±	1.68

* Only 6 specimens.

Results

Effects of implanted hormones. The effects of the presence of the various hormonal compounds on feeding, as measured by engorged weight of the nymphs on the day of drop off, and molting, as measured by the duration of development in days, are summarized in table 5. The most important effects occurred with BSEA-28. When the feeding capsules were examined, few engorged larvae were found feeding, in contrast to the hundreds of fed and feeding larvae usually seen; on one animal, less than 10 fed larvae could be found, and numerous partially fed larvae were seen shrivelled and dead, though still attached, while others were found dead and shrivelled under the stockinet; similar observations were made on the other BSEA-28 treated rabbit, although close to 100 fed or feeding larvae were counted. When the engorged nymphs were collected, only 6 surviving individuals were collected from one of the animals, while 75 unusually small, stunted individuals were collected from the other. The mean weight of the engorged nymphs, 13.53 ± 4.79, was sig- nificantly less than that of the controls. When observed for the number of days required for molting, these nymphs molted in only 15.43 days; some individuals required as little as 8 days to molt. Figure 8 illustrates the distribution of individual molting times. Although the modal points for the two populations differed by only 1 day, it is apparent that the molting curve for the BSEAS-28 group was skewed the left (i.e., shorter molting period), with many individuals molting much earlier than the modal point of 19 days. In contrast, the molting curve for the control group resembles a bell-shaped curve, with the earliest molting at 17 days (individuals), only 3 days earlier than the group modal point of 20 days. Implantation of Methprene and ecdsyterone had no apparent effect on duration of development time to molting. The values recorded were very similar to that of the propylene glycol controls or the untreated controls. Engorged body weight, however, was affected when Methoprene was administered at a dose of 81.3 mg (27.1 mg/kg body weight); the weight of the engorged nymphs increased to 32.98 mg. However, no effect was observed when this compound was administered at a much smaller dose, 41.9 mg (13.97 mg/kg body weight). In contrast to the effect on body weight, there was no effect on molting time, which was almost identical in the population from both animals. Sim-

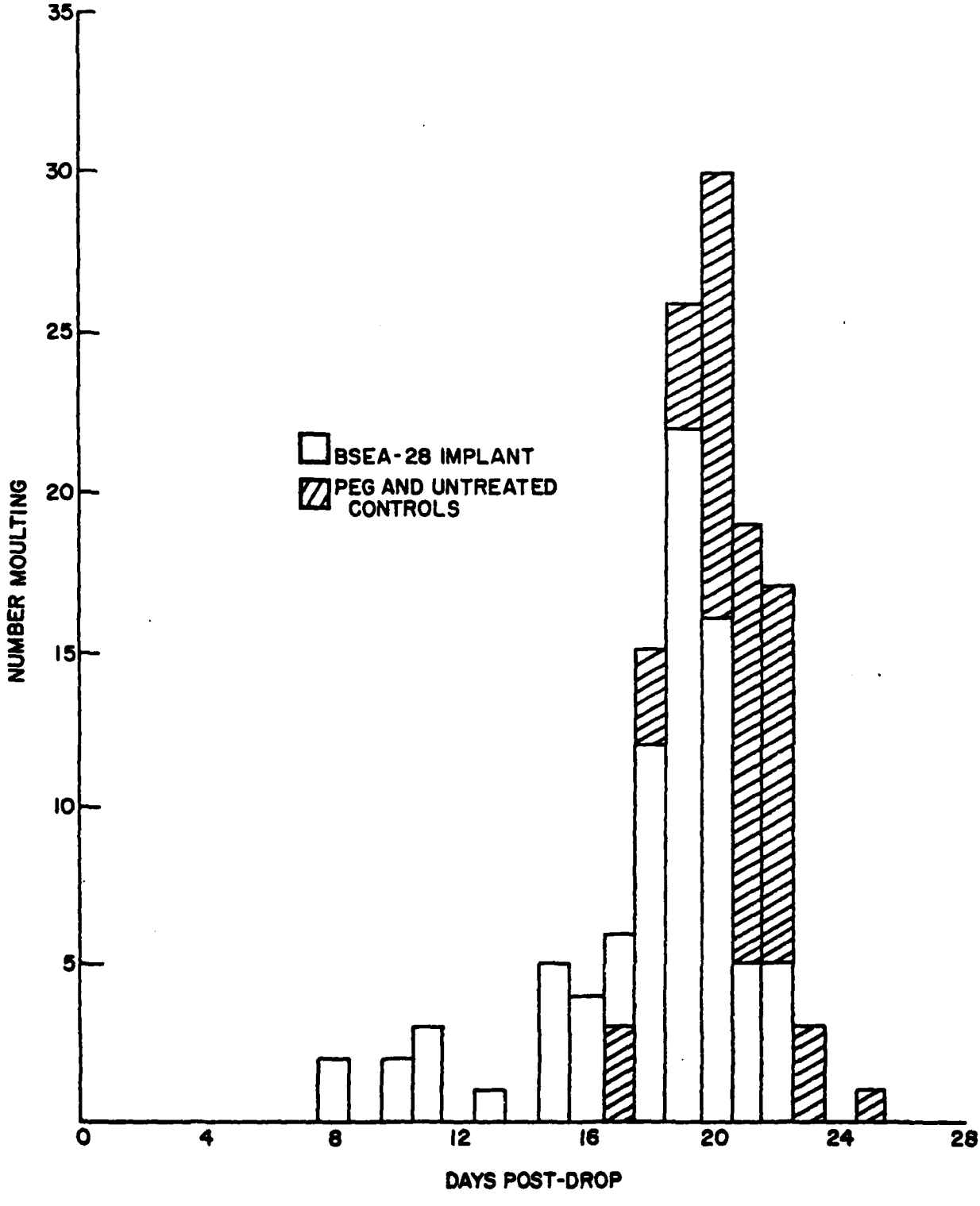

Figure 8. Histogram illustrating molting patterns of H. dromedarii nymphs exposed to the BSEA-28 implant versus the controls.

ilarly, treatment with ecdysterone had no affect on either engorged nymphal tick weight or molting time.

Treatment of engorged H. dromedarii nymphs with ecdysterone, or a combination of ecdysterone and JH I had no effect on the duration of molting in these ticks; nymphs inoculated with the former molted in 20.61 ± 1.70 days; with the latter, 21.26 ± 2.47 days.

The results of GLC assays of H. dromedarii females that emerged from nymphs exposed to the various hormone treatments are summarized in Table 6. The highest concentrations of 2,6-DCP/tick were recorded in individuals from nymphs inoculated with ecdysterone (9.24 ng/tick). Relatively high concentrations were also recorded in females from nymphs treated with both ecdysterone and JH I, only slightly less than in females receiving ecdysterone alone (7.72 ng/tick). Much lower values were observed in females from nymphs fed on Methoprene or beta-ecdysone treated rabbits. No 2,6-DCP was found in males from any of these treatment groups. Assays of the 2,6-DCP in the control populations are still in preparation.

Discussion

The results suggest that one of the compounds, BSEA-28, significantly affected development and molting in H. dromedarii. Both engorged body weight and duration of molting was affected by this material. In addition, numerous larvae died while feeding on one of the two treated rabbits.

The ability of natural ecdysteroids to induce early molting or even supermolting in ticks is well known. Mango (1976, 1979), in her very extensive studies with these materials, demonstrated that ecdysterone would accelerate molting in the argasid tick Ornithodoros porcinus when administered topically or in blood which ticks imbibed through membranes. Doses as low as 1 ug/tick administered topically accelerated molting when administered on the day of engorgement, as did Ponasterone A, a closely related phyto-ecdysteroid. Addition of these ecdysteroids to the in vitro blood meal also accelerated molting. A closely related compound, 22, 25-dideoxyecdysone introduced into the in vitro blood meal at 2 and 3 ug/mg

Table 6. Occurrence of 2,6-dichlorophenol in Hyalomma dromedarii adults that emerged from nymphs exposed to different hormonal treatments.

Treatment	Sex of Ticks	No. of Individuals	2.6-DCP ng/DCP
Ecd. inocul. 10 ng/nymph	females	68	13.09
"	males	26	1.97
Ecd. inocul. (10 ng/nymph) + 10 ug JH	females	71	9.69
"	males	43	3.52
BSEA-28 implant	females	49	10.10
"	males	45	5.03
Ecdysterone implant	females	100	5.80
"	males	105	0.00
Methoprene implant	females	89	6.90
"	males	114	0.00
Prop. glycol implant	females	39	to be done
"	males	47	0.00
Untreated control	females	100	5.57
"	males	to be done	to be done

exhibited a highly lethal effect on the feeding ticks, which died in the process of molting. Estimates of ecdysteroid uptake given by Mango were generally in the range of 1 or 2 ug, to a high as 2.7 ug/tick.

Khalil et al. (in press) observed accelerated molting in H. dromedarii when ecdysterone was applied topically to feeding and fed nymphs. When engorged nymphs were treated with does of 1, 5, 10 or 29 ug beta-ecdysone in ethanol, the mean premolting period was reduced significantly, in some cases to as little as 12 days (controls ranged from 22.8 to 24.5 days). Doses of 10 or 20 ug/tick invariably reduced the premolting period, but high mortality was an important side effect. Occasionally, accelerated molting was observed even with doses as low as 1 ug/tick.

All of the treatments described above required relatively large doses to achieve their effects, exposing the ticks to exogenous ecdysteroids far in excess of their normal body concentrations (picogram or nanogram amounts). In contrast, the ecdysteroid analogue, BSEA-28, appears to be active at much lower doses. Assuming an absolutely uniform rate of release from the implant with 100% passage of the active material over a 6 month period, and retention of all of the molecule in the rabbit's blood without excretion or metabolic degradation, the concentration to which the feeding ticks would have been exposed in a 3.5 kg rabbit would be ca. 4 ug/ml. This value is close to Mango's (1979) effective dose range. If these assumptions are incorrect, which is very probably the case, the actual concentrations can only have been lower. Thus, there is good reason to expect that H. dromedarii is very sensitive to ecdysteroid analogues. In contrast, comparable concentrations of ecdysterone (190 mg/rabbit) had no effect, perhaps because subthreshold values were maintained.

In contrast to the effects of ecdysteroids, JH analogues had no apparent effects on tick development. Methoprene, administered as an implant, also appeared to affect engorged body weight in one instance; when the concentration of active ingredient was increased from 41.9 to 81.3 mg, the engorged body weight of the nymphs increased significantly. However, the duration of the molting period was unaffected and it is probable that this observation, based on one animal, is not meaningful. JH also failed to

affect sex pheromone activity. Although inoculation of ecdysterone increased sex pheromone (2,6-DCP) content (confirming previous findings of Dees et al., in press), addition of JH 1 to the ecdysterone treated ticks did not significantly reduce this effect (9.4 ng/tick in the former, 7.72 ng/tick in the latter).

In summary, ecdysteroids appear to affect H. dromedarii development and sex pheromone activity. The ecdysteroid analogue, BSEA-28, appears to be very potent when administered in a slow release device, reducing body weight and the premolting period. Ecdysterone, when administered directly in very low doses (10 ng/tick) increases sex pheromone concentrations. Juvenile hormone analogues studied thus far have no apparent, consistent effect on these same biological parameters in this species.

VI. Evidence of the Role of the Cheliceral Digits in the Perception of Genital Sex Pheromones in Ticks. (ODU)

Introduction

The chelicerae of ticks serve as the cutting and skin penetrating organs, essential in the process of attachment to hosts. In Boophilus microplus, the cheliceral digits contain sensillae and sensory neurons linked to the cheliceral nerve (Waladde, 1976, 1977; Waladde and Rice, 1977). Experimental evidence obtained by these workers indicated that the chemosensory sensillae were responsive to ATP, glutathione, and other compounds common in vertebrate blood (Waladde and Rice, 1977, 1982). Presumably, these sensillae play an important role in perception of blood meal quality.

In addition to their role in feeding, the chelicerae also function in the process of copulation. Feldman-Muhsam (series of papers) demonstrated that the chelicerae are inserted into the female gonopore and described how these appendages transfer the spermatophore. The insertion of the chelicerae into the female gonopore during copulation may serve to identify chemical or physical stimuli necessary to complete this process. Recently, Sonenshine et al. (1982) demonstrated the existence of a genital pheromone (or pheromones), perceived by male Dermacentor variabilis and Dermacentor andersoni. In these species, the males probe the gonopores of females of either species with their chelicerae, but copulate only with conspecific females.

We were interested in determining whether the cheliceral digits are important in detection of sex pheromones in ticks. This information may be important in determining whether hormones may influence genital sex pheromone activity (or even serve as the pheromones).

Materials and Methods

Ticks. D. variabilis was reared from specimens collected near Richmond, Virginia. Except when feeding, ticks were held in an Aminco-Aire®

Climate Lab (American Instrument Co., Silver Spring, MD) at 27 C ± 1° and 90 ± 2% RH. Immature ticks were allowed to feed on albino rats, [Rattus norvegicus], adults on rabbits [Oryctalagus cuniculi].

Scanning electron microscopy (SEM). For examination of the external surface of the cheliceral digits, the palps of males fed 7 days were excised and the entire capitulum was removed, placed in 70% ethanol, and refluxed in 100% pyridine for 4 hours as described by Waladde and Rice (1977). The cleaned, coated specimens were viewed with a Cambridge Stereoscan 100 scanning electron microscope.

Transmission electron microscopy (TEM). The mouthparts of males fed 7 days were excised and fixed for 2-3 hours in cold (4°C) 4% glutaraldehyde buffered in 0.1 M S-Collidine, pH 7.4. The tissues were washed, were processed by standard EM techniques and embedded in Epon 812. Following hardening, thick (1 um) and thin (0.1 um) sections were cut using an LKB Ultramicrotome III. A diamond knife was used for the thin sections. Thick sections were stained for 3 seconds in a modified azure-methylene blue stain (Richardson, 1960). Thin sections were mounted on formvar-coated copper grids and coated with carbon in a vacuum evaporator. The coated sections were stained with saturated uranyl acetate and lead citrate. The stained sections were viewed with a Hitachi HU 11-B transmission electron microscope.

Experimental procedures. Males attached 7 days were removed from their hosts and segments of their palps or chelicerae excised using microsurgical techniques. In one group, only the 3rd and 4th articles of the right palp were removed; in another, the same articles of the left palp were excised; in another, both the entire right and left palps were removed (as close to the basis capituli as possible). In another group of males, the anterior ends (including the digits, hood, and sheaths) of the right chelicerae were removed; in another, the same components of the left chelicerae were removed; and, finally, in another, the same components of both chelicerae were removed. The numbers treated in each case are given in table 7. Following microsurgery, the treated males were allowed to reattach to their hosts, feed, and recover from the surgical trauma for 1-2 days, detached again, and surviving males were used for the bioassays.

Table 7. Mating responses of sexually active Dermacentor variabilis males following excision of the cheliceral digits or palpal segments.

Parts of appendages excised	No. males treated	No. females	No. trials[1]	O	P	C	% males that copulat.
Right palp (art. 3 & 4)	18	18	22	100.0	77.3	50.0	61.1
Left palp (art. 3 & 4)	12	12	13	92.3	92.3	84.5	92.3
Both palps (entire palp)	14	19	30	100.0	96.7	26.7	57.1
Right chelicera (digits + sheath)*	17	20	35	88.6	40.0	2.9	5.9
Left chelicera (digits + sheath)*	13	14	49	100.0	83.7	1.7	7.7**
Both chelicerae (digits + sheath)*	10	10	10	100.0	100.0	0.0	0.0
Controls	To be added						

* Digits, anterior end of cheliceral shaft, anterior portion of cheliceral sheaths removed.

** Examination of this male after copulation revealed excision of the right cheliceral sheath, but the cheliceral digits were intact.

1 Each male was allowed 3 trials unless it copulated or attached.

Bioassays were done as described by Sonenshine et al. (1977) and Khalil et al. (1981), except that the males were allowed 3 chances to orient (O), position (P), and copulate (C) with each of 3 partially fed females. Thus, each male was allowed 9 trials, unless it copulated or attempted to feed (i.e., attached to the host skin rather than continue mating responses). Controls consisted of untreated males detached from their hosts after feeding for 7 days. Following the bioassays, each male was examined to verify surgical ablation of the appropriate structures.

Results

Ultrastructure. When the cheliceral sheaths and hood were removed from each chelicera, SEM reveals that the external surfaces of the cheliceral digits bear pores, pore-like depressions, minute spurs, and a plate-like structure near the junction of the inner and outer digits.

Transmission EM reveals that the interior of the cheliceral digits bears a lumen containing the cheliceral nerve and its branches. Sections near the tip of the inner digit denticle reveals 3 groups of dendrites similar to those identified by Waladde and Rice (1977). At this level, one dendrite occurred alone. Another group was found which contained 11 dendrites. Both groups of dendrites resemble chemosensory dendrites. A third group contained 2 dendrites, each with the neurotubular arrangement and dense scolopale characteristic of mechanoreceptors. Transverse sections through the outer digit reveled only a single bundle of 13 chemosensory type dendrites.

Proximally, at the level of the junction of the inner and outer digits, the cheliceral sensory dendrites, neurones, and glial cells are found to fill most of the lumen. A large dendrite extends laterally from the cheliceral nerve through a channel in the cuticle wall, to the plate-like sensillum described previously. Numerous microtubules are evident when sections through this neurite are examined, and the dendrite is enclosed in a scolopale. These characteristics are representative of mechanosensory dendrites, in this case, associated with the plate-like sensillum.

Experimental results. The results of the bioassays with males surviving the various treatments are summarized in Table 1. Males in which the 3rd and 4th articles of the right or left palps were removed oriented to sexually active females in almost all cases; most probed the female gonopore (positioning, P) and 50% or more of the trials resulted in spermatophore transfer (copulation). The frequency of successful copulations declined when both palps suffered amputations, but orientation and positioning responses were unaffected. Very different results were observed when the cheliceral digits were removed. Orientation responses were unaffected, but positioning responses were reduced, and copulations failed in virtually all cases. Even those males that attempted to probe the female gonopore did so only for very brief periods, often less than 1 minute, and exhibited hesitating, erratic behavior when they crawled onto the ventral surface of the females. Controls exhibited high frequencies of mating responses in all response categories.

Discussion

The participation of the tick chelicerae in copulation has been described by several workers, notably Feldman-Muhsam and Borut (1971), Moorhouse (1966), and Oliver (1974). That the chelicerae may play an active role in this process, guided by direct sensory stimulation, has not been considered. However, this is suggested by the discovery of chemosensory sensilla capable of detecting compounds such as adenosine triphosphate, reduced glutathione, and others (Waladde and Rice, 1977). As Waladde and Rice (1977) have noted, the chelicerae can no longer be regarded as merely passive cutting tools. We suggest that the chelicerae also serve as the organs which detect the genital sex pheromone(s) and, therefore, are essential to successful copulation. Support for this hypothesis includes the experimental evidence cited in this study, the existence of chemosensory sensilla and neurone innervation in this same species, and the role of genital sex pheromones described in previous studies.

The most important evidence of an active role for the cheliceral digits in copulation comes from the experimental studies. These findings confirm that the intact, functional chelicerae are essential, not only for sperma-

tophore transfer, but for the formation of the spermatophore as well; males in which the digits were excised not only failed to copulate, they also failed to release spermatophores from their own bodies. Clearly, if the chelicerae were merely passive objects, the spermatophores would have appeared and implantation would have been attempted. Other evidence of an active chemosensory role for the chelicerae is apparent in the behavior of the copulating male. Even though the digits had been removed, the males still probed the female gonopores. This response is similar to that observed by Sonenshine et al. (1982) following abrasion, washing, or coating of the female genital surface, i.e., curtailment of copulation. Both findings suggest a chemical mediator, i.e., a pheromone perceived by sensilla on the cheliceral digits.

Comparison of the sensilla and nervous innervation of the cheliceral digits in Boophilus microplus (Waladde and Rice, 1977) and that of D. variabilis reveals a remarkable similarity between the two. Their findings of gustatory functions may also be assumed to apply to other species, especially where the structure of receptors and nervous innervation is similar. Conclusive proof of the role of these structures, however, remains to be discovered.

VII. Comparative Effects of the Antiallatotropin Precocene-2 (P2) on 3 Acarine Species Representing 3 Reproductive Strategies (GSC)

The previous 6-month progress report indicated that attention had shifted from the tick Dermacentor variabilis to the Chicken mite Dermanyssus gallinae. We reported the results of three major experiments, viz. (1) Determination of the most effective concentration of P2, (2) Determination of gonotrophic sensitivity to P2 exposure, and (3) Determination of normality of F_1 offspring from treated females. This report deals with two additional experiments involving precocene and D. gallinae, viz. (4) Effects of time in recovery of P2 treated females and (5) Determination of ability of juvenile hormone (JH3) to reverse P2 effects upon oogenesis and oviposition.

In experiment 4 six experimental and 2 control groups were used. Mites in the experimental groups were exposed to 1 mg. P2 in acetone at sequential times (immediately after feeding to 16 hours after the second blood meal). All mites were fed the 3rd blood meal 7 days after treatment and fed the 4th blood meal 14 days after treatment (DAT). The bioassays included fecundity, hatchability and larval mortality. Differences in egg production following the 3rd blood meal were not significant, although mites in all of the treated groups produced slightly fewer eggs than the controls. Egg hatchability and larval mortality were not significantly different in all groups following the 3rd feeding. There were also no significant differences in the 3 parameters measured among treatment groups and controls after the 4th feeding. The highest average egg production per mite among P2 treated groups was 3.92 exhibited by the 16 hour females which is exactly the same average produced by mites in the acetone control groups. Precocene 2 treatment had significantly reduced fecundity of females after the 2nd feeding and lowered hatchability and viability of larvae. It was surprising that after a period of one and two weeks the treated mites appeared to have recovered regarding the three measured parameters.

Our earlier experiments indicated that P2 had negative effects upon oogenesis, oviposition, and hatchability. Therefore, our fifth experiment was designed to determine whether juvenile hormone (JH3) could reverse the influences of P2 upon oogenesis and oviposition in D. gallinae. Six groups (30 mites/group) were fed twice and then immediately after feeding 4 groups

were treated with 1 mg. of P2. Three of these P2 treated groups also received a JH3 treatment 24 hours after the P2 application. Two of the JH treated groups received the hormone topically, with 1 group receiving .1μg and the other group receiving .5μg of JH. The remaining JH group received a .5μg amount on a filter paper disc. Control groups were acetone-treated or untreated. Adult mortality, oviposition, egg hatchability and larval mortality served as bioassays.

Egg production differed significantly (P=.05) among the treated groups and controls. All of the P2+JH groups, and the P2 group differed from the controls. As expected, maximum egg production was exhibited by the controls, and the lowest egg output resulted from the P2 treated group. Although egg production did increase in the groups treated with the P2 and JH3, overall output remained significantly different (P=.05) from the controls. Both topical and paper applications of JH3 were equally effective as indicated by Duncan's multiple range test. No difference in egg production existed between the groups receiving .1 and .5μg JH3 topically, and the group receiving .5μg JH3 on filter paper disc. The JH3 applications were partially successful in overcoming P2's negative ovipositional effects, however, complete recovery of ovipositional ability was not achieved.

Egg hatchability was normal among all groups except the P2 group, which differed significantly (P=.01). Larval and adult mortality were negligible among all groups.

VIII. Summary

Collaborative investigations on hormone-pheromone interactions were continued with the camel tick, Hyalomma dromedarii, the species of primary interest, and the American dog tick, Dermacentor variabilis.

The major emphasis during the recent project year was focused on natural tick hormones, and their interaction with pheromone activity, the main goal of the project. Special attention was given to ecdysteroids, which had been reported to accelerate sex pheromone activity. The ability of exogenous ecdysterone to stimulate sex pheromone activity was confirmed. The presence of ecdysterone (beta-ecdysone) and, most probably, ecdysone, was determined (by chromatography) in H. dromedarii. Ecdysterone was also found in D. variabilis; ecdysone almost certainly also exists in this species, but could not be detected. Other unidentified ecdysteroids were recognized by RIA, as well as non-polar fractions of unknown identity. The ecdysteroids, and, in a general sense, the entire steroidal component of the two species, are remarkably similar. These findings add to the increasing body of evidence that ecdysone and ecdysterone commonly occur in ticks and are probably universal in the suborder. A comprehensive survey of the various life stages and physiological states was done for the presence of ecdysteroids in D. variabilis. This study included all stages from early embryonic development to the ovipositing female. This investigation is believed to represent the most extensive study of the time course of occurrence of ecdysteroid in ticks. This information may be expected to provide a basis for determining when to expect onset of different physiological events (e.g., onset of sex pheromone activity) as well as data which may be useful in predicting effective doses of hormonal analogues used in tick control studies. Other important new evidence demonstrated the biosynthesis of ecdysterone from cholesterol, and the apparent increased sensitivity of the ecdysial process to relatively low concentrations of ecdysone analogues. In contrast to the accumulating evidence of the effects of ecdysteroids on tick physiological processes, treatment of ticks with juvenile hormone analogues (JH I) had no apparent effects. However, studies with mites indicated a response of the reproductive system to the anti-

allatotropin, Precocene-2 (P2), which may be interpreted as evidence of a JH-like system in these acarines. When JH (JH3) was applied, partial recovery of ovipositional ability occurred.

Another important study concerns the role of the chelicerae in the perception of the genital sex pheromones. New evidence demonstrated the presence of an extensive system of nerve branches, with dendrites innervating both chemosensory and mechanosensory receptors on the cheliceral digits. Experimental evidence is presented which describes the role of the cheliceral digits in the perception of the genital sex pheromone(s). These findings provide additional evidence of a pheromone regulated mechanism which acts to guide the male to the gonopore and facilitates species-specific mate recognition.

IX. Future Plans

The project will continue with efforts to characterize the hormonal mechanisms that affect sex pheromone activity in ticks. Efforts to identify the various ecdysteroids present in the ticks at times when they may have an opportunity to influence sex pheromone activity will be continued, especially those found to react in the radioimmunoassay. Preliminary efforts have indicated two ecdysteroids, which may be polar metabolites of ecdysterone, or may be active molecules. We hope to collect sufficient tick material (from mass rearing) to facilitate chemical studies, especially mass spectroscopy, that will permit precise molecular determinations.

The biosynthesis and site(s) of synthesis of the tick ecdysteroids will be investigated in this forthcoming study. Preliminary evidence indicates biosynthesis of ecdysterone from radiolabelled cholesterol. We plan to expand on this discovery, to determine the efficiency of the process, its byproducts, and the periods of active synthesis. Included will be a study to determine whether labelled hormones accumulate in the pheromone glands and interact in some way with the sex pheromone. Extracts of tick synganglion, gonads, pheromone glands, and other organs will be examined for evidence of natural ecdysteroid production and accumulation of labelled hormone. These studies may contribute to an understanding of the synthetic pathways involved in hormonal activity, information which is essential in the search for hormonal antagonists or inhibitors.

The role of juvenile hormones in stimulating or inhibiting sex pheromone activity will also be investigated. Isolation of JH-like fractions from tick tissues will be attempted. Studies with exogenous JH, especially JH3, will also be done.

Work on the effects of precocene-2 on acarine JH hormones will be continued. JH reversal studies will also be done. These studies have provided promising indications of natural JH systems in mites and ticks.

X. Publications and Manuscripts

The following is a list of all new publications, papers previously cited which have since been published, manuscripts previously cited which have been accepted, and new manuscripts in preparation. All of the articles cited have been produced, accepted, or published since the last progress report.

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1. Sonenshine, D.E., P.J. Homsher, G.M. Khalil, and S.N. Mason. 1982. Dermacentor variabilis and Dermacentor andersoni: genital sex pheromones. Exper. Parasitol. 54:317-330.

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11. Khalil, G.M., D.E. Sonenshine, R. Milton Silverstein, P.J. Homsher, and H. Hoogstrall. Sex pheromones of Ixodidae. IBID (in press).

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